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EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

XI. Antibodies in the Blood after Recovery from Epidemic Influenza.

By PETER K. OLITSKY, M.D., and FREDERICK L. GATES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, September 20, 1922.)

In earlier papers of this series we have reported the observation of specific antibodies against *Bacterium pneumosintes* in the blood serum of experimentally infected rabbits. Thus, specific agglutinins were detected after the intratracheal injection of the active agent of the nasopharyngeal secretions of influenza patients,[1] after the intratracheal or intravenous injection of living cultures of *Bacterium pneumosintes*,[1] and after the subcutaneous injection of killed cultures of this microorganism.[2] It is evident from the results of these experiments that the reaction of the animal body to *Bacterium pneumosintes* involves the production of the antibodies that are commonly recognized by serological tests. We have no reason to suppose, therefore, that the mechanism of protection against this organism, of which we have evidence in the immunity reactions already described,[3] differs from that which comes into play in the case of infections with aerobic pathogenic organisms.[1]

Bacterium pneumosintes has been found only in the nasopharyngeal secretions of influenza patients, in the early hours of the disease. The question arises whether the presence of the organism induces a specific reaction on the part of the host. Evidence of such a reaction is to be sought in the presence of antibodies in the blood. As has already been stated, the opportunity to test the blood serum of patients convalescent or recently recovered from influenza during the epidemic

[1] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 553.
[2] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxvi, 685.
[3] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 1.

waves of 1918–19 and 1920 was lost for lack of a suitable antigen during the earlier periods of our experiments. The return of influenza to New York City in January and February, 1922, again afforded us an opportunity to collect specimens of human serum for serological examination. The results of these tests are reported in this paper.

Method.

The specimens for examination were collected with sterile precautions and the sera were stored at 4°C. without preservative. It was anticipated that low dilutions of serum would be required in the tests and therefore specimens which showed any hemolysis or any opacity due to fats were discarded. We have pointed out that *Bacterium pneumosintes* is a stable organism with a narrow acid flocculation zone and that even prolonged immunization of rabbits yields an immune serum of relatively low titer (1:16 to 1:32). It was not to be expected that antibodies against *Bacterium pneumosintes* would be demonstrable in high dilutions of human serum, whatever the significance of their presence might be. In order to obtain results as clear-cut and unequivocal as possible we had recourse to a method of agglutination recently described by Northrop and De Kruif.[4] These authors show that the addition of serum or other proteins to suspensions of certain thoroughly washed bacteria, suspended in buffer solutions at various hydrogen ion concentrations, widens their zone of acid agglutination and changes the isoelectric point of the bacteria to that of the added protein. Practically, this means that microorganisms, washed and suspended in a buffer solution at a hydrogen ion concentration just to the alkaline side of the isoelectric point of an immune serum, are rendered more sensitive to specific agglutinins and consequently respond more readily by flocculation than would ordinarily be the case. If the hydrogen ion concentration of the buffer solution is properly chosen, the specificity of the agglutination reaction is not impaired. Thus, Table I illustrates the reactions of two strains of *Bacterium pneumosintes*, tested in immune and in normal rabbit sera by the methods described below.

[4] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 655.

Northrop and De Kruif's observations were applied to our problem as follows:

Anaerobic cultures of *Bacterium pneumosintes* in ascitic fluid or dextrose broth with a fragment of rabbit kidney were used in amounts of 1 cc. to seed 100 cc. Florence flasks of *B. coli* broth[5] which were incubated under a petrolatum seal for 3 to 5 days, according to the growth obtained. The organisms were concentrated with the centrifuge, killed by heat at 56°C. for 30 minutes, and washed three times with distilled water. This concentrated stock suspension was standardized by opacity[6] and diluted to a staphylococcus equivalent of 1 to 1.2 billion organisms per cc.[2] with Northrop and De Kruif's buffer solution[7] at pH 6.3. The buffered suspension was preserved with thymol crystals.

TABLE I.

Specificity of Agglutination Tests in Universal Buffer Solution at pH 6.3.

Animal.	Agglutinogen.					
	Strain 17.			Strain 34.		
	Serum dilution.					
	1:10	1:20	1:40	1:10	1:20	1:40
Rabbit A (immune).	+++	+++	++	+++	++	+
" B "	+++	+++	++	+++	+++	++
" C (normal).	−	−	−	−	−	−
" D "	−	−	−	−	−	−

In the tables +++ indicates complete agglutination with a clear supernatant fluid; ++, almost complete agglutination with a slightly clouded supernatant fluid; +, distinct agglutination with a clouded supernatant fluid; −, no agglutination or serum flocculation; and 0, no test.

[5] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 813. The *B. coli* broth was prepared as follows: Nutrient broth, made of beef infusion, with 1 per cent peptone and 1 per cent dextrose, in lots of 100 cc., was seeded with 0.1 cc. of a 24 hour culture of *B. coli communis*, grown in the same medium. As soon as the faintest haze due to growth became visible, usually after 1 to 3 hours at 37°C., the flasks were layered with petrolatum, and placed in the Arnold sterilizer at 100°C. for 30 minutes. This treatment killed the organisms and established anaerobic conditions under the petrolatum seal. The rapidly cooled flasks were then ready for inoculation with *B. pneumosintes*.

[6] Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.

[7] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639.

Most of the agglutination tests with human serum were made at a serum dilution of 1:10. Less frequently dilutions of 1:20 and 1:40 were included also. The dilutions were made by adding undiluted serum in amounts of 0.1, 0.05, and 0.025 cc., delivered from a fine, sterile, capillary pipette attached to a 0.5 cc. tuberculin syringe, to 0.9, 0.95, and 0.975 cc. of the buffered bacterial suspensions in 1 by 10 cm. tubes. In each instance the tip of the pipette was immersed in the suspension, since formed drops do not measure accurately such small amounts of fluid.

Control tubes were also set up of the buffered bacterial suspension and salt solution, without serum, and of the serum and buffer solution without bacteria. These last controls were required because a serum was occasionally found which was itself precipitated at pH 6.3. Such serum precipitation might simulate bacterial agglutination, or sweep the bacteria out of suspension, and the few samples which showed this reaction were therefore discarded.

The experimental and control tubes were then shaken and incubated in a water bath for 16 to 20 hours at 43°C. before macroscopic reading.

Two strains of *B. pneumosintes* were used in the tests. Strain 17 was obtained directly from the filtered nasopharyngeal secretions of an influenza patient in the first (1918–19) wave of the epidemic. Strain 34 was obtained from the affected lung tissue of a rabbit injected with the nasopharyngeal washings of a patient in the third (1922) outbreak of influenza.

Agglutination Tests.

Three series of tests were performed: the first with the serum of convalescent patients 10 to 20 days after the onset of influenza; the second with the serum of other patients 3 to 5 months after recovery; the third with serum of persons who gave a history of influenza during the first two waves (1918–19 or 1920). With these sera were included, as controls, specimens from a number of persons from whom no history of influenza could be elicited.

The results of the first series of tests are collected in Table II. A survey of the table shows that the serum specimens of twelve of the fourteen persons examined shortly after an attack of influenza contained specific agglutinins for *Bacterium pneumosintes*. When tested, those sera which agglutinated the organisms completely or almost completely in a dilution of 1:10 showed distinct agglutination in a dilution of 1:20 also. Attention may be called to the fact that the 1919 and the 1922 strains of *Bacterium pneumosintes* reacted with these sera in an identical manner. This antigenic identity of old and new strains has already been pointed out.[8]

[8] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxvi, 501.

The results of the tests on the three samples of serum from Case 37 are of particular interest. $2\frac{1}{2}$ months before an attack of uncomplicated influenza, the serum of this person was obtained as a normal control and found to contain no agglutinins for *Bacterium pneumosintes*. Then the attack of influenza supervened and 10 days later

TABLE II.

Agglutination Tests with the Serum of Convalescent Influenza Patients, Obtained 10 to 20 Days after Onset.

Case No.	Agglutinogen.		Buffer solution + serum.	Length of time after onset.
	Strain 17.	Strain 34.		
	Serum dilution.			
	1:10	1:10	1:10	
				days
29	+	+	−	17
30	++	++	−	10
33	+++	+++	−	11
33	+++	+++	−	17
34	++	++	−	13
35	+ (1:5 ++)	+ (1:5 ++)	−	15
37	−	−	−	Normal.*
37	+	+	−	10
37	++	++	−	89*
38	− (1:5 +)	− (1:5 +)	−	20
42	−	−	−	13
43	++	++	−	18
44	+	+	−	10
45	+	+	−	10
46	+	+	−	17
47	0	−	−	10
48	++	++	−	16

* Included for comparison.

agglutinins had appeared in the patient's serum. A third serum specimen, obtained 89 days after the onset of influenza, showed the persistence of agglutinins in the blood.

This persistence of agglutinins in the serum is illustrated by the second series of tests, made with sera obtained from five other persons who had recovered from influenza 3 to 5 months previously. The

results of these tests are shown in Table III. They were controlled with specimens of immune and normal rabbit serum, positive human serum (Nos. 33 and 37), buffer solution plus serum (shown in the table), and bacterial suspension plus salt solution.

That the agglutinins against *Bacterium pneumosintes* which are found in the blood serum of persons convalescent or recently recovered from influenza do not persist indefinitely thereafter is shown by the results of tests in a third series. In this group were included serum specimens from ten persons who gave a history of an attack of influenza during the epidemic waves of 1918–19 or 1920. In three instances the

TABLE III.

Agglutination Tests with the Serum of Persons 3 Months or More after Recovery from Influenza.

Case No.	Agglutinogen.		Buffer solution + serum.	Length of time after illness.
	Strain 17.	Strain 34.		
	Serum dilution.			
	1:10	1:10	1:10	
				mos.
49	+	+	−	4
50	+	+	−	5
51	+++	+++	−	3*
52	++	+	−	5
54	++	+	−	3½

* Had recurrent attacks during the three waves of influenza.

illness was complicated by a secondary pneumonia. In none of these sera were specific agglutinins demonstrated.

It was not easy to select as controls a series of persons who had unquestionably not had influenza during the past 3½ years. Most of the controls were chosen from patients, mainly suffering from chronic cardiac ailments, who had spent months or years in institutions and who gave no history of influenza during the recent prevalence of the disease. Serum specimens from twenty-two such persons gave negative results in agglutination tests identical with those described above.

We have, therefore, to contrast to these twenty-two negative controls, agglutination tests with serum specimens from nineteen persons with a history of influenza in the 1922 wave. Seventeen of these specimens specifically agglutinated two strains of *Bacterium pneumosintes*, one obtained during the first epidemic wave (1918–19) and the other obtained in 1922. On the other hand, the sera of ten persons who had influenza during the 1918–19 or 1920 waves of the epidemic contained no similar agglutinins.

The findings in the case of one other person are difficult to classify. They concern one of us whose serum, obtained in 1921, contained no agglutinins against *Bacterium pneumosintes*. A recent (1922) specimen, however, in a dilution of 1:10, showed a + agglutination of both old and new strains. During this interval he has been working with *Bacterium pneumosintes* almost constantly and it is not unlikely that an accidental mild infection may have occurred. Whether this happened, or whether one of several transitory ailments was a mild or abortive influenza, cannot be stated. Hence it is not possible to classify him either as an influenza patient or as a normal control.

Precipitin Reactions.

Precipitin tests were also performed with a number of the available sera as a check on the specificity of the agglutination reactions. The precipitinogen was prepared from a dialysate culture[1] of a 1922 strain of *Bacterium pneumosintes* (Strain 36) several months old which was filtered through a Berkefeld N candle to obtain a clear antigen. This antigen was carefully layered on clear, non-hemolyzed, undiluted serum specimens by drawing first the filtrate and then the serum up into a glass capsule of 2 mm. bore. This method produces a sharp line of junction in which the slightest cloud is easily detected between the clear columns above and below. The prepared capsules were incubated in a vertical position for 1 hour at 55°C. and placed over night in the ice box at 4°C. Controls for the antigen consisted of normal, and of known positive rabbit serum from actively immunized animals.

Precipitin tests were performed on the sera of ten patients, collected 10 to 20 days after an attack of influenza, in which specific agglutinins

for *Bacterium pneumosintes* had been found. In each of these tests a clearly marked zone of opacity developed at the juncture of serum and *pneumosintes* antigen. With the sera from Case 37 (Table II) the precipitin reactions paralleled those of agglutination. The specimen obtained before the attack gave a negative test; the two samples obtained after the attack reacted positively. Two positive precipitin reactions occurred among the five sera obtained from 3 to 5 months after recovery from influenza. The serum specimens of seven normal control persons gave negative results.

SUMMARY AND CONCLUSIONS.

Although *Bacterium pneumosintes* is a stable organism which agglutinates only in low dilutions of the serum of actively immunized rabbits, the serological reactions of this microorganism have always been found to be consistent and specific. The results of the agglutination and precipitation reactions described in this paper indicate that the serum of normal persons does not contain demonstrable agglutinins or precipitins for *Bacterium pneumosintes*. By contrast, agglutinins have been demonstrated in the serum of seventeen persons among nineteen who were examined from 10 days to 5 months after recovery from epidemic influenza. The serum of ten persons who had influenza, followed in three instances by pneumonia, $2\frac{1}{2}$ to $3\frac{1}{2}$ years before, proved negative. In one instance the appearance of specific agglutinins against *Bacterium pneumosintes* was found to be coincident with an attack of uncomplicated influenza. In twelve of fifteen instances in which agglutinins were found, precipitins against *Bacterium pneumosintes* were demonstrated also. It is noteworthy that these antibodies may persist in the blood at least 5 months after recovery from the disease.

THE CULTIVATION OF ANAEROBIC TREPONEMATA ON THE SURFACE OF BLOOD AGAR PLATES.

By FREDERICK L. GATES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 15 TO 17.

(Received for publication, October 3, 1922.)

In the course of recent experiments with *Bacterium pneumosintes*, isolated by Olitsky in 1918 from the nasopharyngeal secretions of influenza patients,[1] it was found that this strict anaerobe, which had heretofore been cultivated only in the depths of a solid medium, or in fluid media under a petrolatum seal, could be grown in colony form on the surface of rabbit blood agar in a Brown anaerobic jar.[2] Since *Bacterium pneumosintes* was isolated in the medium used by Noguchi for the cultivation of anaerobic treponemata, and appeared to have similar nutritive requirements, these observations suggested that the method might succeed with spiral organisms also. There were available several strains of *Treponema pallidum*, *Treponema calligyrum*,[3] and *Treponema microdentium* which had been maintained in culture for periods of 8 to 11 years.[4] These cultures seemed especially suitable for test because of their saprophytic character and long adaptation to an artificial environment.

Preliminary experiments were therefore undertaken with four strains of treponemata, two of *pallidum*, and one each of *calligyrum* and *microdentium*. Although the observations are incomplete, definite results were obtained, apparently for the first time with anaerobic spiral organisms, and since the experiments cannot be continued at present, the results so far obtained are reported to indicate the possibilities of the method for the cultivation of anaerobic treponemata in colony form.

[1] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.

[2] Brown, J. H., *J. Exp. Med.*, 1921, xxxiii, 677.

[3] Noguchi, H., *J. Exp. Med.*, 1913, xvii, 89.

[4] These strains were obtained from Dr. Noguchi.

Method.

The anaerobic jar described by Brown[2] is a modification of those devised by McIntosh and Fildes[5] and by Smillie[6] and utilizes the reaction of oxygen and hydrogen in the presence of palladinized asbestos. The catalyzer is heated by a resistance coil in a wire-screened chamber, which, on the principle of the Davy safety lamp, obviates all danger of explosion. The continuous introduction of hydrogen and application of heat rapidly carry the reaction to completion and produce a condition within the jar that is highly favorable to strict anaerobes. Within a few hours medium containing blood changes from its usual crimson color to the cyanotic hue of reduced hemoglobin.

The medium used consisted of ordinary nutrient agar, made with beef infusion peptone broth, to which 5 to 7 per cent of fresh, sterile, unheated, defibrinated rabbit blood was added just before the plates were poured. This medium was seeded with several drops of an ascitic fluid-rabbit kidney culture of treponemata, spread with a platinum loop. The plates were incubated in the anaerobic jar at 37°C. for 6 to 9 days before examination. Subsequent transfers were made from single colonies, and incubated for periods up to 39 days.

Cultivation Experiments.

By the method described, one culture of *Treponema pallidum* was grown in colony form for ten generations, the other for eight, and the strain of *Treponema calligyrum* was grown for five generations, when the experiments had to be discontinued for the time being. The *microdentium* culture was not successfully carried beyond the first transfer.

The primary growths of all four cultures produced zones of complete hemolysis in the blood agar and decolorized the medium in certain areas to a pale straw color, but the presence of even a small amount of precipitate from the fluid cultures used for seeding obscured the presence of definite colonies. In subsequent generations of *pallidum* and

[5] McIntosh, J., and Fildes, P., *Lancet*, 1916, i, 768.
[6] Smillie, W. G., *J. Exp. Med.*, 1917, xxvi, 59.

calligyrum characteristic colonies appeared which could be easily recognized and differentiated morphologically. These colonies require separate descriptions.

Treponema pallidum.—The two cultures of *Treponema pallidum* used were labelled ZA and R.[7] The hemolysis produced by Culture ZA is shown in Fig. 1. On close examination by transmitted light the zones of most complete decoloration were found to be centered around minute, elevated colonies overlying a brown-stained area in the medium. At the edges of the hemolyzed areas a delicate mantle of spreading growth was observed. It was clearly visible only under a hand lens as a slight roughening of the agar surface. This growth was invisible by transmitted light. Transplants made separately from the raised centers and from the periphery of the growths developed two diverse types of colonies, one raised and sharply circumscribed, the other flat and spreading.

Colonies of the spreading type are shown in Figs. 2 and 3. The colony in Fig. 2, 9 days old, may have a small raised colony in the center. Fig. 3 is of a culture in a later generation, 39 days old, and shows the rate of growth in that interval. The spreading colonies were flat, moist, slightly rough, and had a delicate fimbriate edge formed by slender fingers of growth which coalesced in the body of the colony by lateral extension, and often spread distally to cover large hemolyzed areas of the medium.

The growth consisted of a dense felted mass of treponemata morphologically similar to *Treponema pallidum.* The single forms were delicate, usually with regular, rectangular spirals, and frequently showed a double contour or pseudobranching (Figs. 4 and 5). In older cultures long, thready, less regular forms occurred, tapering off gradually at the ends (Figs. 6 and 7). Most of the spirals were not motile, or showed only the lazy twisting motion typical of old cultures of *pallidum.* The rapid boring progress of active young *pallida* was not observed.

[7] Culture ZA was obtained from Dr. Zinsser in 1915 and was the Strain A described by Zinsser, Hopkins, and Gilbert (Zinsser, H., Hopkins, J. G., and Gilbert, R., *J. Exp. Med.*, 1915, xxi, 213). Strain R was cultivated by Dr. Noguchi in 1911 from the testicle of a syphilitic rabbit (Noguchi, H., *J. Exp. Med.*, 1911, xiv, 99).

The raised colonies derived from the same ZA culture differed widely from the spreading ones. They were sharply outlined, raised, convex, translucent, with an entire edge, and produced a faint brown pigmentation of the hemolyzed and nearly colorless medium. The surrounding area of hemolysis extended about 1 mm. from the edges of the colonies (Fig. 8). The raised colonies were usually discrete, and from 0.1 to 1 mm. in diameter. They made confluent streaks along the tracks of the inoculating loop but did not spread to fresh areas. Single treponemata from the raised colonies are shown in Figs. 9 and 10. They too were usually regular in morphology, with fine rectangular turns, but they were less delicate than the spreading type, appearing to have twice their diameter.

Further work is required to determine whether these two types of treponemata were both present in the original culture or whether one type resulted from the other by mutation. While the spreading colonies remained true to type for eight generations, cultures of the raised type, seeded from carefully chosen single colonies, have repeatedly been found to contain some spreading colonies also.

Culture R of *Treponema pallidum* gave somewhat similar results. Raised and spreading colonies developed from this culture also. Both types were hemolytic. The raised colonies and the treponemata of which they were composed appeared to be identical with the corresponding elements of Culture ZA. But the spreading colonies, in pure culture, consistently differed slightly from those already described. This difference appeared at the edges of the growth (Figs. 11 and 12) where the fimbriæ were much larger and more irregular than those of the colonies of Culture ZA. Fig. 11, like Fig. 2, is of an early generation 9 days old. The raised centers of some of the colonies may be due to an incomplete separation of the types. Fig. 12, of a pure culture 39 days old, may be compared with Fig. 3. The treponemata in the spreading colonies of Culture R were not distinguishable morphologically from those of the spreading ZA culture (Figs. 13 to 15).

Transplants of the spreading colonies of Cultures ZA and R into tubes of semisolid ascitic fluid-rabbit kidney medium, overlaid with 3 cm. of liquid petrolatum, produced characteristic and indistinguishable growths. In shake cultures the agar was diffusely clouded

up to 1.2 to 1.5 cm. from the surface. At this point the growth stopped abruptly in a more intense brownish line. In stab cultures a spreading cloud of growth developed along the line of puncture, penetrating the medium to a distance of 5 mm. The stab line was not clearly marked. 1.2 to 1.5 cm. below the upper surface a narrow brownish cloud of growth spread completely across the tube. Similar cultures from carefully chosen raised colonies were not noticeably different from those of the spreading type. The upper limit of growth, marked by a brown line, was approximately the same. Usually the stab line was more opaque. It was surrounded by a similar cloud penetrating the agar, but it is not certain that these cultures were free from treponemata of the spreading type.

Treponema calligyrum.—The culture of *Treponema calligyrum* also produced hemolysis in blood agar plates. The growth spread over the inoculated surface in colonies consisting of conical centers surrounded by a confluent plateau (Figs. 16 and 17). These colonies were apparently homogeneous; at least, no separation of types was accomplished by repeated transfers.

The growth was colorless and delicate, so that a hand lens was required to bring out its details. Treponemata from the *calligyrum* plates are shown in Figs. 18 and 19. They are seen to be more like the raised than the spreading types from the *pallidum* cultures.

Treponema microdentium.—This organism produced areas of hemolysis in the blood agar medium, which, unlike those underlying the other cultures, had a diffuse grayish opacity. Although these opaque areas were rich in treponemata, no growth on the surface could be seen and transfers of a number of primary cultures were unsuccessful. The organisms grew into the medium for one generation only, presumably on nutritive elements carried over with the original inoculum.

DISCUSSION.

It is evident from these preliminary experiments that certain anaerobic treponemata may be cultivated in colony form on the surface of suitable media in an anaerobic jar. As far as I am aware, this is the first time, but one, that spiral organisms have been grown in surface colonies.

In 1921, Twort,[8] describing experiments performed several years previously, reported the development of colonies of a strain of spirochetes originally obtained from a lymph gland of a mouse. When sown on the surface of a specially prepared medium, in tubes capped with gutta-percha and incubated aerobically at 37°C. for 9 days, a delicate growth of spirochetes appeared. The colonies were small, 1 to 2 mm. in diameter, with a dull, rough surface and an irregular outline, usually heaped up in the center. In color they were a light fawn, later a rich brown. Secondary colonies in older cultures were larger, smoother, and of the same brown color. Twort states that no growth was obtained under anaerobic conditions.

The turns of the single organisms were fairly close, varying from 1 to 30 in number. No flagellum was demonstrated. In old cultures the spiral forms were less numerous, and in their place appeared numbers of round bodies, from a large coccus to a small yeast cell in size, containing chromatin granules. Subcultures developed spirochetes.

No growth was obtained in broth containing 10 per cent of fresh, sterile rabbit blood, but if this medium was previously incubated for 48 hours, a definite growth, with hemolysis, occurred. Such cultures were filterable.

Rabbits and pigeons appeared to be immune to this organism. A mouse, injected subcutaneously, developed sores, but the spirochetes could not be recovered from the lesions. These experiments were interrupted by the war so that the method of cultivation could not be tested with *Spirochæta pallida* and the culture of mouse spirochete unfortunately died out.

The successful experiments of Twort with an aerobic spirochete, and the present series with anaerobic treponemata, indicate that the spiral organisms which had previously only been cultivated in the depths of solid or fluid media can produce characteristic surface colonies by which they may be separated and identified. A few attempts to cultivate *Treponema pallidum* on anaerobic blood agar plates directly from the testicular lesion of syphilitic rabbits have been unsuccessful. But it is probably only a matter of experiment to establish conditions under which direct isolations may be made. It should then be possible to identify *Treponema pallidum* and other treponemata by colony formation, to separate them more easily in pure culture, and to obtain suspensions of the spiral organisms free from the proteins of the medium, and therefore suitable for serological and immunological tests.

[8] Twort, F. W., *Lancet*, 1921, ii, 798.

SUMMARY.

By a method of cultivation that proved successful with *Bacterium pneumosintes*, characteristic surface colonies of old saprophytic strains of *Treponema pallidum* and *Treponema calligyrum* have been obtained. The presence of two types of colony in the *pallidum* cultures requires explanation, but the fact of colony formation may point the way to new methods of isolation and identification and to the utilization of suspensions of treponemata, free from foreign proteins, for serological and immunological purposes.

EXPLANATION OF PLATES.

PLATE 15.

FIG. 1. Hemolysis produced in blood agar by a surface growth of *Treponema pallidum*. × 1.

PLATE 16.

FIG. 2. Colony of spreading type, 9 days old, from Culture ZA of *Treponema pallidum*. × 10.

FIG. 3. Fimbriate edge of spreading growth 39 days old, Culture ZA. × 10.

FIGS. 4 to 7. Treponemata from spreading colonies, Culture ZA. × 1,000.

FIG. 8. Colonies of raised type, Culture ZA. × 10.

FIGS. 9 and 10. Treponemata from raised colonies, Culture ZA. × 1,000.

PLATE 17.

FIG. 11. Colonies of spreading type, 9 days old, from Culture R of *Treponema pallidum*. × 10.

FIG. 12. Fimbriate edge of spreading growth 39 days old, Culture R. × 10.

FIGS. 13 to 15. Treponemata from spreading colonies, Culture R. × 1,000.

FIG. 16. Colonies of *Treponema calligyrum* 9 days old. × 10.

FIG. 17. Surface growth of *Treponema calligyrum* 39 days old. × 10.

FIGS. 18 and 19. Treponemata from surface colonies of *Treponema calligyrum*. × 1,000.

(Gates: Cultivation of anaerobic treponemata.)

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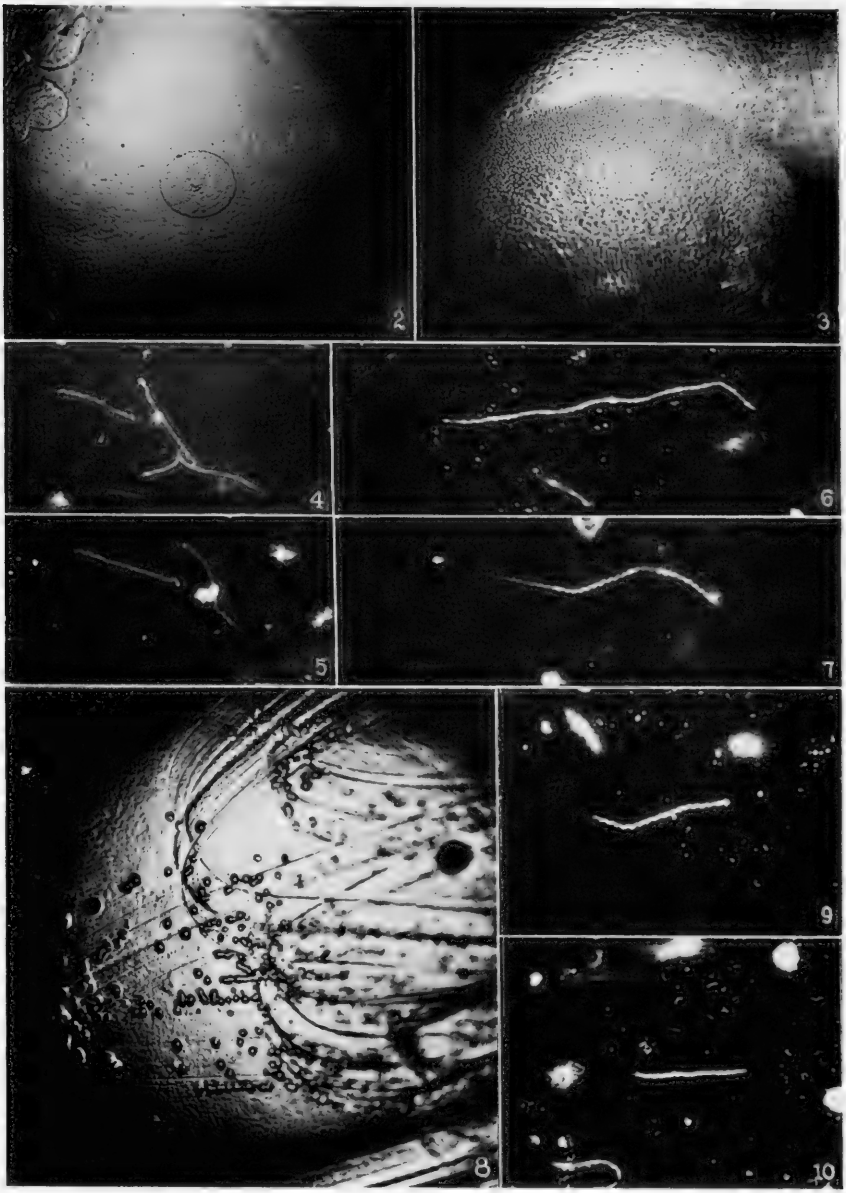

(Gates: Cultivation of anaerobic treponemata.)

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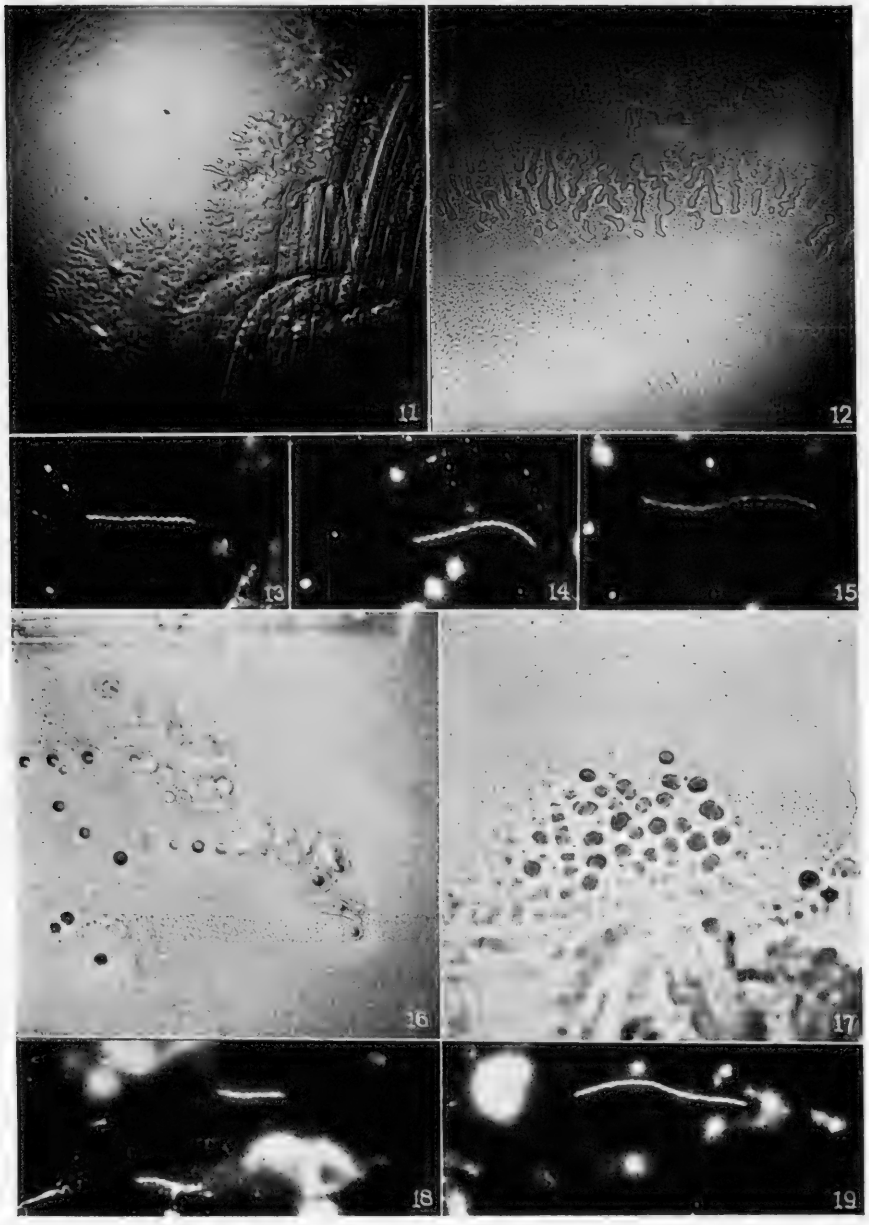

(Gates: Cultivation of anaerobic treponemata.)

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STUDIES ON THE CHANGES PRODUCED BY ROENTGEN RAYS IN INFLAMED CONNECTIVE TISSUE.

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PLATES 18 TO 22.

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INTRODUCTION.

Although Roentgen rays have been used for more than 20 years in clinical medicine and despite the extensive literature, very little is as yet known about the action of this form of energy upon living substance. Not only is the nature of the action not clear, but there are lacking even systematic experimental investigations on the common histological changes in the cells and tissues after treatment with x-rays. These considerations induced me to undertake the experiments described below in 1919. The material can by no means be considered complete, but as the work has been discontinued the results obtained so far will be described in the present article.

The literature on the biological action of x-rays will not be discussed at length as this has been done by previous authors.

It is generally admitted that, of all the cells of the vertebrate organism, the greatest sensitiveness towards x-rays is displayed by elements possessing a particularly high degree of reproductive capacity and, on the other hand, showing no finally settled morphology and function. Accordingly, the most striking results have been obtained with sex cells, eggs and spermatozoa. X-raying of these elements leads, in the subsequent development, to various monstrosities and eventually may cause the death of the embryo. If the sexual glands in the body are exposed, they are "sterilized;" *i.e.*, the egg and sperm cells perish, whereas the nutritive cells, the follicle and Sertoli cells, remain alive (Krause, Reifferscheid, and others).

On the other hand, among the somatic cells, the lymphocytes, being indifferent, non-granulated, lymphoid cells of the blood and the blood-forming organs, are especially sensitive towards the rays. After exposure of lymph nodes, spleen, thymus, etc., the lymphocytes are rapidly destroyed (Krause, Fromme, Soper, Grasnick, Hartmann, and others).

The Roentgen tube, radium, whose γ-rays closely correspond to x-rays, also mesothorium and thorium X seem to give in all the reported cases almost identical results. The differences are not important; for example, it is the myeloid and not the lymphoid tissue, that seems to be especially sensitive towards thorium X (da Silva Mello, Zoellner).

It is also well known that of the different types of epithelial tissue the germinal layer of the epidermis and the hair follicles are considered the most sensitive; in clinical work with x-rays this is always to be noticed and this property of the epidermis often causes ulcerations of the skin and alopecia (Rost).

References concerning other tissues will be made in the course of the following discussion.

As a rule, even in small doses the rays have a damaging and inhibiting influence upon the living substance, manifesting itself in various degenerative changes in the respective cells. Yet in recent years observations have been made which seem to make it probable that, under certain conditions, a stimulating effect might be produced by using very small doses. Development of plants may perhaps be stimulated by small doses of x-rays. Haecker and Lebedinsky, Packard, Baldwin (1919–20, 1920), and others have seen a stimulating influence of x-rays on the development of animal eggs. Murphy and his collaborators also emphasize the stimulating action of small doses of x-rays on the lymphocytes of the circulating blood and the lymphoid organs.

What parts of the living cell are chiefly affected by the rays is not precisely known, as special cytological investigations in this direction are lacking. But it has been shown since the classical studies of O. Hertwig and his school that, of all the cell organs, the nucleus and especially the chromatin content of the latter are first affected. This must be the chief cause of the abnormalities in development after exposure of germ cells to the action of rays. This specific sensitiveness of the nuclear chromatin, as indicated by fragmentation of the nuclei, etc., has since been confirmed by many authors (Hoffmann, Packard, Payne, Rost, and others).

As we do not yet possess a systematic knowledge of the cytological and histological changes produced in cells by the action of x-rays, it seems natural that even such thorough work as, for example, that of Hartmann on the influence of x-rays on the blood-forming organs in amphibian larvæ should yield mere commonplace results in the sense of a general inhibition of lymph cell formation. In the general uncertainty one meets from time to time quite unexpected and startling facts, as, for example, the remarkable sensibility of the epithelium in the crypts of the small intestine, discovered by Warren and Whipple (1922, *a*, *b*) and McQuarrie and Whipple. Profound and rapid injuries of the intestinal function and a peculiar Roentgen ray intoxication are caused, whereas the lymphoid elements of the small intestine seem hardly to be affected.

In studying the changes in the cells and tissues caused by the action of x-rays, one has to discriminate between instances in which the

rays are acting upon the whole organism, and those in which only a limited part of the latter is exposed and the action is only local. It is evident that in the first case the results must be incomparably more complicated and far more difficult to control than in the second.

My intention was to study the influence of the x-rays upon the cellular inflammatory process in the loose connective tissue. The inflammatory changes in the connective tissue have been for many years the subject of my investigations (Maximow, 1902, 1903, 1904, 1905, 1906). This problem has a certain interest in itself, as the changes produced in the connective tissue elements by the action of the rays are unknown. Furthermore, its clinical importance is not to be underestimated, as the loose connective tissue, distributed all over the body, is inevitably involved in every case of therapeutic use of the rays and its behavior must play an important part in every case of wound healing, of tumor development, etc., and must directly determine to some extent the issue of the pathological process. According to Ribbert the changes produced in the connective tissue by Roentgen rays are responsible for the origin of the Roentgen carcinoma. Fraenkel believes that the loose connective tissue is to be looked at as a single large endocrine system; he cautions against using high doses of rays in treating carcinoma, since the extremely important defense reaction of the connective tissue is apt in such a case to be imperilled or weakened.

Material and Methods.

The experiments were performed on rabbits and, for inducing aseptic inflammation, the method previously described (Maximow, 1902) was used in which flat rectangular celloidin blocks, with slits cut parallel to their surface, are introduced into the subcutaneous or intermuscular loose connective tissue of the abdominal wall. Generally the appropriate place in the abdominal wall was exposed on the day after the introduction of the celloidin blocks and the exposure was repeated once a day for a certain period. The animal was stretched out, with the back downward, and covered with a tightly fitting, duly bent lead sheet of 3 mm. thickness. The lead had a round opening of about 4 cm. in diameter, corresponding to the position of the celloidin blocks, which are easily felt through the skin. Above the opening the Roentgen tube was placed with an aluminum plate of 0.5 mm. thickness as filter. The tubes used were of the Gundelach or Müller type. The distance between the anticathode and the surface of the skin was as a rule about 14 to 15 cm. The length of the daily exposure was on the average 15 minutes.

For determining the dose Kienboeck's test was used, the dose employed every time being about 12 of Kienboeck's quantimetric units, the so called x.

The first series includes eight cases of this kind; in one of them only one exposure was made, in two others there were two, in one four, in one five, in one ten, and in two fifteen exposures. After the last exposure a period of 5 to 27 days was allowed to elapse; in one instance this period was prolonged to 60 days. At the end of this time the animal was killed and the part of the body wall containing the blocks was fixed, after the skin had been carefully removed and the tissue stretched over a cork frame. The pieces of tissue were fixed in Zenker-formol or in some cases Champy's liquid, embedded in celloidin, and cut in serial sections parallel to the surface. For staining, eosin-azure or iron-hematoxylin was used, and after Champy's fixation the method of Kull. Identical celloidin blocks were always introduced simultaneously on the opposite side of the abdominal wall for control. These remained without exposure to x-rays and were fixed together with the exposed blocks.

In most cases on the exposed area of the skin alopecia of higher or lesser degree developed. In the three rabbits in which the exposure was repeated ten or fifteen times, a superficial inflammation of the skin could be observed. Ulceration never occurred and, even in the animal which had been exposed fifteen times and had lived 27 days after the last exposure, the initial inflammation again subsided.

In one instance simultaneously with the introduction of the celloidin bodies 2 cc. of lithium carmine were injected into the same place in the connective tissue; in another 2 cc. of trypan blue were used for the same purpose.

In a second series of experiments, in three rabbits, a certain area of the abdominal wall was exposed on each of 3 days before the introduction of the celloidin blocks; two exposures were made, separated by an interval of 5 minutes. In two of the rabbits celloidin blocks were introduced into the previously exposed place 5 days after the exposure, in the third rabbit 10 days after the exposure, and left there for 3, 10, and 25 days respectively. Control blocks were also introduced into other, normal places in the abdominal wall.

Lastly, the influence of x-rays on scar tissue was tested; for this purpose in two animals celloidin bodies, introduced in the abdominal wall 15 days previously, were exposed five times during 5 days to a dose of 12 x.

The process of the aseptic inflammatory reaction in the connective tissue consists of a regular series of many closely connected phenomena. An important part is played here by local sessile elements, the fibroblasts and resting wandering cells, as well as by wandering elements, special leucocytes, lymphocytes, and polyblasts, coming for the most part from the blood vessels. The exposure to x-rays of all these elements with their different sensitiveness towards the rays, together with the constant changing of the sensibility according to

their different stages of development, must evidently produce very complicated results. This complexity is still more increased by the fact that the action of the rays does not at once become manifest, and furthermore by the constant arrival of new cells from the blood vessels into the connective tissue. A preparation from a block several times exposed will thus contain, on one hand, cells which have been acted upon by the rays to a greatly varying degree and have been living and functioning for different periods of time after their last exposure, and, on the other hand, cells on which the x-rays did not act at all, but which had to exist for different lengths of time in the inflamed tissue, which had been previously affected by the rays.

Macroscopically, the tissue surrounding the foreign bodies and exposed to the rays shows only one more or less distinct difference in comparison with the control side—the inflamed tissue directly adjacent to the foreign body seems to be edematous and softer, and, especially after repeated exposures, the celloidin block appears surrounded by a more or less thick layer of a semifluid substance. For this reason careless dissection may easily permit the celloidin block to slip out of the surrounding tissue capsule. In the oldest case, killed 60 days after the last exposure, no difference was found in the macroscopic aspect of the exposed and the control tissues. In both the foreign body was closely wrapped in dense scar tissue.

In all the animals the results described below were in principle quite identical. The differences noted were only those of degree and depended on the number of exposures and on the stage of the inflammation. The effect of the x-rays was very distinct on the 5th day after a single exposure of a celloidin body introduced the day before; it was still more distinct after two exposures. The greater the number of exposures, the more distinct become the changes in comparison with the control side, and in the two rabbits exposed fifteen times they were pronounced on the 8th and 15th days after the last exposure.

The Granulation Tissue and the Exudate Layer Surrounding the Foreign Body.

As an illustration of the earliest visible changes an example will be described in which the celloidin body had been exposed twice to a

dose of 12 x and had been fixed 11 days after the second exposure. The aseptic inflammation had thus had 13 days in which to develop.

In sections made parallel to the surface of the control block typical granulation tissue with beginning scar formation is found (Fig. 2). The fibroblasts (*Fbl*) have considerably increased in number and constitute a new tissue layer, enveloping the foreign body. Their flat cell bodies contain regular oval nuclei with dust-like chromatin granules and with the typical large nucleoli. Between the cell bodies are located the newly formed, thin, densely arranged, collagenous fibers (*C*). Everywhere between the fibroblasts are scattered the polyblasts (*Plb*). For a description of the latter the reader is referred to former papers (Maximow, 1902, 1903, 1904, 1905, 1906). Small, recently emigrated, lymphocyte-like polyblasts are rare. Most of these cells have already reached the resting stage and have flat, elongated or slightly branched cell bodies and typical, dark, irregularly folded nuclei. Since in this instance trypan blue was injected together with the blocks, the protoplasm of most of the polyblasts contains, on eosin-azure slides, distinct granules of a grayish blue color. Granulated special leucocytes are very scarce.

The newly formed granulation tissue is expanding outwards without sharply defined limits into the surrounding common normal loose connective tissue. Towards the surface of the celloidin is a thin layer of multinucleated syncytium-like giant cells, lymphocytes, phagocytic polyblasts of varying size, granular special leucocytes, and necrotic cell remains chiefly derived from special leucocytes. In the interior of the celloidin body the process of tissue invasion is going on—fibroblasts, singly and in groups, phagocytic polyblasts in different stages of development, innumerable, partly degenerated, special leucocytes, and outgrowths of capillary vessels are penetrating into the clefts.

The tissue enveloping the exposed blocks has a different aspect (Fig. 1). In fact, a newly formed layer of connective tissue is entirely lacking. The foreign body is surrounded by a rather thick exudate layer of peculiar structure; it has in a fixed preparation a loose net-like structure and gives at first sight the impression of clotted fibrin (Fig. 4). This can be easily confirmed by using Weigert's fibrin stain; the fine fibrils of the net are stained dark blue. Thus, the

celloidin block is embedded in a mass of clotted fibrinous exudate. It is the semifluid mass seen, as described above, on macroscopic examination and causing the ready dislodgment of the bodies exposed to x-rays. In the meshes of the net ameboid cells are scattered everywhere—special leucocytes (Fig. 4, *Lkc*), easily distinguishable by the polymorphic nuclei and the specific granules, stained bright red by eosin-azure, and polyblasts of different stages of development—every possible transition between the smallest lymphocyte-like cells (*Lmc*) and the large macrophages, crowded with trypan blue granules (*Plb*). Some of the polyblasts have branched pseudopodia or appear elongated and have a worm-like shape. Degenerating polyblasts with shrunken pycnotic nuclei are found here but rarely. Degenerating special leucocytes (*Lkc'*) are, on the contrary, numerous and show disintegrating nuclear debris and partly dissolved, partly scattered granules. They are the chief object of the phagocytic activity of the polyblasts mentioned above (*Plb'*).

On the surface of the celloidin of the x-rayed blocks are dense masses of small lymphocyte-like and large phagocytic polyblasts and great quantities of special granular leucocytes. Degenerating forms are especially common here, chiefly among the special leucocytes.

At the periphery of the layer of fibrinous exudate a wide, clear, highly edematous tissue zone is seen (Fig. 1). It is not newly formed, but old, deeply changed connective tissue. The thick, wavy collagenous fibers (*C*) appear swollen and the spaces between them are filled with clear liquid, containing in many places fibrin nets. In the direction towards the fibrinous layer these collagenous fibers are lost in the clotted mass. Towards the surrounding tissue they gradually pass over into the apparently unchanged collagenous fibers.

The fibroblasts in this edematous tissue layer lie widely separated from each other (Fig. 1, *Fbl*). Their number seems not to have increased at all and mitotic figures are not found within them. The cell body, as can be easily seen by referring to Fig. 1, *Fbl*, is no longer thin and flat, but appears considerably thickened and enlarged and shows either a polygonal or fusiform shape with long tapering ends. The protoplasm is strongly basophilic and stains dark after eosin-azure. The nucleus is also considerably enlarged; in the majority of the cells it keeps its regular oval form unchanged, but the chroma-

tin granules inside are coarser and the nucleoli appear to be much larger. In many fibroblasts the nucleus is showing unmistakable signs of fragmentation, of constriction into single parts of very different size (*Fbl''*). Near the nucleus in the protoplasm often a clear space is to be seen—a similarly hypertrophied attraction sphere.

The polyblasts in the edematous zone are much more numerous than in the capsule of the non-exposed control body, described above; they are scattered in irregular groups among the fibroblasts and look quite different. Their appearance seems to correspond to a much younger stage of development. For the most part they are still quite similar to lymphocytes; they are small, round, ameboid cells with an eccentric kidney-shaped nucleus (Fig. 1, *Lmc*). In the protoplasm the progressive accumulation of trypan blue granules is clearly seen. They are freshly emigrated lymphocytes on their way to be transformed into polyblasts (*Plb*). The minority of them are larger cells of very different, mostly fusiform shape with sharply outlined, sometimes dentated contours, with a dark, irregularly folded nucleus and with a varying quantity of trypan blue granules in the protoplasm; these are doubtless the local resting wandering cells on the point of being transformed, by a process of rounding off and mobilizing, into polyblasts (*Rwc*). In the polyblasts mitoses are not to be found; under normal conditions also, as is well known, they divide only rarely in the field of inflammation.

The edematous tissue contains also special granulated leucocytes in considerable numbers (Fig. 1, *Lkc*); they seem thus to continue their migration out of the blood vessels into this tissue even in relatively late stages.

The pathological cell forms just described become more and more rare as the normal connective tissue is approached, but one can find them occasionally as far away as between the adjacent muscle fibers.

In the clefts of the celloidin bodies no trace is to be found of an ingrowth of young connective tissue and vessels; one sees here only innumerable, mostly degenerated, special leucocytes and also some degenerated polyblasts in various stages of development.

In the cases in which the introduced celloidin bodies were exposed many times to the average dose of 12 x, a relatively high total dose, the changes are naturally much more accentuated. In two, in which,

after introduction of the bodies, fifteen exposures were made during 15 days and the tissue was fixed 8 and 27 days respectively after the last exposure, so that the total duration of the inflammation was 24 and 43 days respectively, the capsule of the control bodies is composed of common dense scar tissue with fibroblasts, containing fibroglia fibrillæ, and with a dense feltwork of newly formed collagenous fibers. Between the fibroblasts sessile polyblasts with polymorphous nuclei and often long and branched protoplasmic processes are scattered at regular intervals. This scar tissue adheres almost directly to the surface of the celloidin, and between the two are seen only few hypertrophic polyblasts and single, flat, multinucleated giant cells. In the more distant layers, especially in the second animal, nets of newly formed capillaries are seen, surrounded by groups of plasma cells and by pigmented sessile polyblasts.

In the clefts of these control preparations a fully developed connective tissue is already present. It consists of large fibroblasts with fibroglia fibrillæ, of sessile, polymorphous polyblasts with irregular nuclei, and of networks of new blood vessels with large groups of plasma cells.

In the exposed preparations on the surface of the celloidin a layer of partly degenerated small and large polyblasts and special leucocytes and for the most part degenerating giant cells are seen. The layer of the fibrinous exudate is still thicker than before. In the first rabbit it has the same foamy or net-like structure as before and contains, besides a few, mostly degenerated, leucocytes, relatively scarce, actively ameboid, chiefly small, lymphocyte-like polyblasts. In the second, 42 day case, it appears already slightly thickened and its net-like structure is no longer definitely outlined; now it seems to consist of dense, roughly fibrillated trabeculæ and irregular pieces and contains numerous, mostly small, ameboid, phagocytic polyblasts and special leucocytes. Among these cells one can find, especially now, many degenerating forms, and in some places the dense fibrinous mass is overcrowded with free pseudo-eosinophilic granules, coming from the degenerated and destroyed special leucocytes.

In the first case no real neoformation of tissue can be seen as yet. The exudate is surrounded just as before by the highly edematous tissue layer, which, however, is more extensive, spreading far between

the adjacent muscle fibers and showing still greater changes (Figs. 5 and 6).

The number of fibroblasts is little increased. As before, they lie far removed from one another. Rarely one succeeds in finding abnormal mitotic figures in them (Fig. 3, *x*); such divisions certainly can lead only slowly to real cell multiplication.

The hypertrophy of the fibroblasts has now become very marked, especially in the second case (Figs. 5 and 6, *Fbl*). With their strongly basophilic, flat, giant cell bodies and their numerous processes running in different directions and often branching, they resemble large nerve cells. The nuclei, mostly oval or kidney-shaped, and occupying an eccentric position, have attained a quite excessive size. Their interior is filled with numerous coarse, darkly stained chromatin granules partly covering the nucleoli, and contains sometimes large intranuclear vacuoles (Fig. 5, *Fbl'*). Whereas mitoses, as mentioned above, are extremely rare, amitotic constrictions of the nuclei are common (Figs. 5 and 6, *Fbl''*). The giant nucleus undergoes in such cases a constriction into two or more parts of very irregular size and shape. These parts, as a rule, occupy a peripheral position in the cell body, whereas in the middle of the latter a group of crowded centrioles is seen even after the eosin-azure stain, lying in a clear space, the sphere.

In the second rabbit there seem to appear in the fibroblasts the first signs of an altogether faint productive reaction; in some places they become arranged in flat layers of net-like texture and produce in their protoplasm distinct fibroglia fibrillæ.

Of neoformation of the collagenous fibrillar intercellular substance not much can as yet be seen. The clear edematous liquid between the hypertrophied fibroblasts still seems to contain only the old preexistent wavy collagenous fibers (Figs. 5 and 6, *C*). They appear partly swollen, partly dissociated into fibrillæ, and become lost towards the celloidin block, as they enter the fibrinous exudate.

The changes in the fibroblasts as above described must surely have been seen at least in part by earlier authors. Gassmann (1898–99, 1904) has made similar statements. As regards the hypertrophy of the cells under the influence of x-rays, Wickham may be quoted. According to him hypertrophy of various cells and, in the first instance, of their nuclei, is common after treatment with x-rays. He

finds it especially in malignant tumors, whose cells die in the condition of colossal enlargement. Edema and swelling of the collagenous fibers are occasionally mentioned in the papers of Gassmann (1898–99, 1904), Unna, Rost, and others. How far these changes can be brought into connection with the so called fibrinoid degeneration of connective tissue of Neumann is difficult to decide.

Whereas in the control preparations of the later stages the pseudo-eosinophilic special leucocytes are practically missing, they are still numerous in the exposed inflamed tissue (Figs. 5 and 6, *Lkc*). Thus, it has to be admitted that new cells of this kind are constantly arriving from the blood vessels. Many of them degenerate and are destroyed, spreading their granules throughout the tissue, or are engulfed by polyblasts (Fig. 5, *Plb'*). These granular cells proved to be especially numerous in the second of the above mentioned two cases, in which 27 days had elapsed after the fifteenth exposure.

As regards the polyblasts, they are also much more numerous than in the control preparations. Most of them belong still to the youngest lymphocyte-like variety (Figs. 5 and 6, *Lmc*). The relatively rare larger forms (*Plb*) evidently must be looked upon as the mobilized, local, resting wandering cells. Some of the latter still contain the deeply staining granular inclusions, typical for these cells in the rabbit. Their development, which in the control preparations has proceeded very far or has even been completed, appears to be in abeyance or checked, so that they all remain in a state corresponding to that of much younger stages of development. In the second case only a few of the larger polyblasts seem to tend toward quiescence, assuming a fixed, flat or elongated shape. Phagocytosis, especially in relation to the degenerated special leucocytes, is energetically continued by the polyblasts. As a result of this phagocytosis one may find in many places smaller and larger polyblasts, containing numerous acidophilic pseudo-eosinophilic granules in their protoplasm. Such elements sometimes may simulate myelocytes.

In the edematous tissue of the second of the two instances referred to above, the plasma cells are numerous (Figs. 5 and 6, *Plc*). Everywhere they, together with lymphocytes, common polyblasts, and special leucocytes, surround in large groups the enlarged capillaries. Among them many can be seen degenerating. On the other hand, once in a while, one finds mitotic figures in them.

In the clefts of the exposed blocks in the two last cases described there is no neoformation of tissue to be found. No fibroblasts are seen here, only living or degenerated leucocytes and polyblasts and their debris. In the second case the majority of the polyblasts in the clefts were transformed into large spherical macrophages, containing large numbers of peculiar needle-shaped crystalloids, deeply stained with eosin-azure, besides engulfed special leucocytes and their remains.

In the specimen of longest duration, in which the celloidin blocks were fixed 60 days after the fourth (and last) exposure, no distinct changes could be found in the tissue surrounding the foreign body in comparison with the control preparations. The celloidin block was surrounded by dense, tough scar tissue of usual structure. There could be noted only the presence of relatively numerous resting polyblasts in degenerated condition, with shrunken and disintegrating nuclei.

Changes in the Blood Vessels.

In all the experiments the capillaries in the edematous tissue layer were found to be enlarged to a greater or less degree; no special changes in the endothelium could be noted, except an occasional slight swelling. Of the other vessels, in the small veins only were distinct changes in the endothelium observed (Fig. 7). The cells appeared much swollen and were protruding knob-like into the lumen. The nuclei showed an exceedingly conspicuous fragmentation and underwent constriction into irregular vesicular parts (*End*).

A similar swelling of the endothelium in the blood vessels of the skin after exposure to x-rays has been described before by Gassmann (1898–99, 1904) and Linser. Such intensive changes in the other coats of the larger vessels and their obliteration, as described by Gassmann, I could not find. But that the endothelium of the blood vessels is in general very sensitive towards the Roentgen rays, apart from the presence of constant edema, can be inferred also from the observations of Bagg, who saw, after injection of radium emanation into pregnant rats, extravasations in the subcutaneous tissue of the embryos.

Changes in the Muscles.

A phenomenon regularly observed in all the experiments was the change caused by the x-rays in the muscular tissue of the abdominal wall, situated above or below the exposed celloidin blocks.

Corresponding data are to be found in the literature. Gassmann (1898–99, 1904) was probably the first to note in Roentgen ulcers in rabbits a peculiar degeneration of the striped muscle fibers. He has seen irregular shrinking and thinning of the fibers, with separation of the fibrils from each other, loss of the parallel position and partly of the cross-striation, resulting in transformation into a homogeneous mass; the nuclei multiplied and giant cell-like syncytia were formed. Between the muscular fibers he found connective tissue rich in nuclei and infiltrated with round cells, polymorphonuclear leucocytes, and plasma cells. Brief references are to be found also in the work of Grasnick and Fromme to dissociation of muscle fibrillæ and loss of cross-striation.

In my material distinct changes in muscular tissue are present as early as 11 days after the second of two ordinary exposures of 12 x each. In the two rabbits in which fifteen exposures were made, they naturally are much more accentuated, although still distributed very unevenly. In the first case, in which the preparations were fixed 8 days after the fifteenth exposure, the muscle fibers nearest the celloidin body (Fig. 8, *a*, *b*, *e*, *f*) are widely separated from one another by the edematous tissue with its swollen collagenous fibers (*C*). In some of them the typical picture of coagulation necrosis can be seen—highly swollen, thickened portions, where the longitudinal as well as the cross-striations are missing and the whole fiber is transformed into a homogeneous mass, partly broken up into pieces of various size (Fig. 8, *e*, *f*). The sarcolemma and the muscle nuclei may be still intact in such places (*f*); in many cases, however, the sarcolemma proves to be absent and the necrotic substance is attacked from many sides by ameboid phagocytic polyblasts and gradually resorbed (Fig. 8, *a*, *Plb*). The same fiber may show in its further course apparently normal structural conditions. In other fibers a gradual disappearance of the myofibrillæ is seen, either through the whole length of the fiber or only in some places. The fibrillæ lose their regular parallel arrangement, become separated from one another, and gradually disappear. At first their cross-striation remains unchanged (Fig. 8, *b*), but afterwards the Q-discs become indistinct and only the Z-discs remain (Fig. 8, *c*). Meanwhile the sarcoplasm accumulates at the periphery of the shrinking bundles of the myofibrillæ, under the sarcolemma, and causes here irregular bulgings on the external contour of the fiber (*c*). In such places the manner in which the Z-discs reach the sarcolemma and become attached to the latter.

so that it appears constricted and the fiber acquires a typical festooned outline, is clearly seen. During this gradual involution of the contractile substance, just as in other instances of muscle atrophy, the nuclei may also change their position and character; they often come to lie deep in clefts between irregularly separated fibrils (Fig. 8, *b*), sometimes even in the axial part of the fiber, and secondly, they undergo amitotic division (Fig. 8, *Am*), so that their number may increase considerably and they may become arranged in long rows.

In the second case, in which the foreign body was fixed 27 days after the fifteenth exposure, the muscle fibers adjoining the body present a still higher degree of change (Fig. 9). They are widely separated from each other by abundant, highly edematous connective tissue, containing many leucocytes (*Lkc*), polyblasts (*Plb*), and plasma cells (*Plc*). The swollen muscle fibers, in the advanced coagulation necrosis as described above, are no longer seen, and seem to be completely resorbed. The majority of the fibers are thin, shrunken bands of sarcoplasm (*k*), with scarce, irregularly arranged fibrillæ without any trace of cross-striation; they contain muscle nuclei, single or crowded in heaps (*N*); remains of a sarcolemma can be made out only in some few places. Often in the course of one and the same fiber, areas can be found with changes of very different degree, so that parts with a relatively well preserved general structure and especially with typical cross-striation alternate with parts that, with their crowded nuclei and their slightly granular or vacuolated protoplasm with scarce fibrils, resemble giant cells.

It is clear that the changes of the muscle fibers closely correspond to those described by Gassmann (1898–99, 1904).

Inflammation in Previously Exposed Connective Tissue and the Action of X-Rays upon Scar Tissue.

In the three rabbits, in which, as stated above, the celloidin blocks on one side of the abdominal wall were introduced in areas previously exposed several times to a double dose of x-rays, comparison with the control preparations of the other side showed changes absolutely corresponding to those described above for the usual experiments.

Especially in the first two animals the same fibrinous exudate occurred around the foreign body, the same edema, the same hyper-

trophy of the fibroblasts, and the same inability of the latter to divide (Fig. 3, *Fbl*). Sometimes in the fibroblasts abnormal mitosis could be detected (*x*). Furthermore, the tissue was also overcrowded with abnormally numerous leucocytes (*Lkc*) and mostly young lymphocyte-like polyblasts of hematogenous origin (*Lmc*), whereas the local resting wandering cells seemed hardly changed (*Plb*). In the rabbit in which the inflammation lasted only 3 days and lithium carmine had been injected, together with the foreign bodies, into the connective tissue, all the polyblasts, even the youngest lymphocyte-like cells, which surely must have come from inside the blood vessels, contained distinct granules of carmine.

In the two animals mentioned above, in which scar tissue had been exposed, there were found no changes in the tissue elements resulting from the exposure.

DISCUSSION AND CONCLUSIONS.

The action of x-rays upon inflamed tissue manifests itself in the first place by a considerable depression of the usual reaction on the part of the fibroblasts. Under normal circumstances these elements begin to divide mitotically during the first 24 hours and soon form a layer of new connective tissue, surrounding the foreign body. After treatment with x-rays they remain idle, do not multiply at all, or start very late and often the division is abnormal. They undergo a high degree of pathological hypertrophy of protoplasm and nucleus. Instead of mitosis often amitotic constrictions appear in the nucleus. The capacity for collagen formation seems also to be lost.

Simultaneously with these changes of the fibroblasts an intensive edema of the connective tissue surrounding the foreign body is to be noted and in the immediate neighborhood of the latter a thick layer of net-like clotted fibrinous exudate is formed.

No distinctive qualitative changes could be found in the leucocytes and polyblasts. Degeneration was present here only to the same extent as in common aseptic inflammation. But first the rate and the duration of the emigration of all the cells coming from the blood were increased, and secondly there was always a distinct delay in the

process of the common transformations usually undergone by the polyblasts on the field of inflammation. The transformation of the polyblasts into fixed resting forms seems above all to be delayed. Therefore, even in late stages, the tissue is overcrowded with granular special leucocytes and with mostly young, lymphocyte-like polyblasts, whereas in the early stages the local resting wandering cells only slowly undergo mobilization.

Furthermore, in the blood vessels swelling of the endothelial cells with fragmentation of the nuclei and, in the striated muscles, degeneration of the fibers can be detected. In the latter there occur partly typical coagulation necrosis, partly atrophy, accompanied by loss of striation, separation of fibrillæ from one another, relative increase of sarcoplasm, and amitotic division of nuclei.

What the ultimate result of all these changes would be, is as yet not clear. In the case of longest duration, in which 60 days had elapsed since the last exposure, no distinct difference could be found between the exposed and control preparations. Thus one might believe that the cell injuries caused by the x-rays, and above all the inability of the fibroblasts to multiply and to elaborate collagen, are again repaired in due time. However, my material is decidedly inadequate in this respect and several cases of long duration should be examined.

It is surprising that the results obtained seem not to agree with the predominating views on the action of x-rays on cells. Apart from the endothelium of the blood vessels, of all the cells present in the field of inflammation the fibroblasts undoubtedly are to be considered as the elements most highly differentiated in a specific sense. I have shown that, as a rule, they do not round up in inflammation and do not produce ameboid cells, but remain unchanged in morphology and, through mitotic division, give rise to the new connective tissue. On the other hand, there can be no doubt that the lymphocytes and the polyblasts are to be looked upon as relatively indifferent cells, endowed with great prospective potencies of development. Thus it might be expected that just the lymphocytes of the inflamed area would be affected in the first place by the rays, as they are in the blood-forming organs, and that the fibroblasts, on the contrary, would be refractory.

But the facts have proved that the most conspicuous and constant changes concern the fibroblasts. They are paralyzed for a long time and made unable to build up new tissue. The fibrinous exudate and the edema might perhaps also depend partly on a direct injury of the colloidal intercellular substance, partly on changes of the endothelium of the blood vessels, cells which are again to be considered as highly differentiated.

Noteworthy signs of degeneration could not be found in the lymphocytes and polyblasts. But here again the necessary early stages 1 to 3 days after the last exposure were not available; it is possible that the emigrated lymphocytes are destroyed by the x-rays rapidly, in an explosive manner, in 24 to 48 hours, as in the lymph nodes or the thymus, or as described by Pautrier in the chronically inflamed tissue of the skin in mycosis fungoides. Their remains might be quickly resorbed and after the last exposure new lymphocytes would have time to emigrate out of the blood vessels and to pass to the tissue. However, if we take this for granted, there remains another inexplicable fact, concerning the local resting wandering cells—their close genetic relationship with the lymphocytes is beyond doubt and yet exposure to x-rays does not seem to affect them. In this connection it may be stated that Soper found that the reticulo-endothelial apparatus, whose cells correspond to the resting wandering cells, is stimulated by small doses of x-rays and paralyzed by large doses. For deciding these problems further investigations are necessary.

The classical researches of O. Hertwig and his school have proved beyond doubt that of all the parts of the cell, the nucleus with its chromatin is affected most by the rays. It is believed that the nuclei in mitotic division are especially sensitive (Holthusen) and that the nuclei of exposed cells lose, in the first place, their capacity for normal mitosis; they either do not undergo division at all or they show pathological mitoses (Grasnick, Gaskell, and others). The observations described above fully coincide with these statements. The nuclei of the hypertrophied fibroblasts attain an enormous size, contain an abnormal amount of chromatin, are sometimes vacuolated, and appear paralyzed and incapable of mitosis or divide abnormally. Perhaps the reason that the fibroblasts in the present experiments are so

strongly affected by the x-rays is that they are the only cells which are preparing for mitotic division directly after the introduction of the foreign body, whereas the lymphocytes and polyblasts only rarely divide in the field of inflammation. The fibroblasts thus are becoming especially sensitive towards the rays, whereas for example in the scar tissue, where they remain quiescent, they are not affected.

As the inflammatory changes in the normal skin after exposure to x-rays are not known sufficiently from the histological standpoint it would be promising to study the action of x-rays on the normal loose connective tissue.

It is evident that the changes in the cells of the inflamed area, chiefly in the fibroblasts, but also in the muscle fibers, under the influence of Roentgen rays, are the result of complicated interrelations between two different agents, first, the inflammation stimulus, and, second, the radiant energy. Neither agent alone in the doses used is able to produce the changes observed. Only the combination of both gives the results described above. It seems to be immaterial, to a certain degree, which of the two stimuli is applied first—whether the foreign body be introduced in previously exposed tissue, or the latter be exposed after the introduction of the body—in both cases practically the same results have been obtained.

The strong inhibitory and deleterious influence of x-rays on inflamed connective tissue ought to be always kept in mind in the therapeutical use of this kind of energy, especially in cases of malignant tumors, in which the local connective tissue in most cases is found in a state of inflammatory irritation.

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EXPLANATION OF PLATES.

All the figures represent sections of the tissue surrounding the celloidin blocks and cut parallel to the surface of the latter. They have been drawn from slides, stained with eosin-azure. A Zeiss achromatic immersion lens $\frac{1}{12}$ and a compensating ocular 4 have been used.

C, collagenous fibers; *End*, endothelium; *F*, fibrin; *Fbl*, fibroblasts; *Fbl'*, giant fibroblasts; *Fbl''*, fibroblasts showing amitotic division of the nuclei; *L*, lumen of a blood vessel; *Lkc*, special (pseudo-eosinophil) leucocytes; *Lkc'*, degenerating special leucocytes; *Lmc*, emigrated small lymphocytes (young polyblasts); *Plb* polyblasts; *Plb'*, polyblasts with remains of engulfed special leucocytes in their protoplasm; *Plc*, plasma cells; *Rwc*, resting wandering cells undergoing transformation into large polyblasts; *Smc*, smooth muscle cells; *V*, blood capillaries.

PLATE 18.

FIG. 1. Inflammation of 13 days; two exposures of 12 x each.

PLATE 19.

FIG. 2. Inflammation of 13 days. Same animal as in Fig. 1; control.

FIG. 3. Inflammation of 3 days in an area of connective tissue which had been previously exposed on 3 consecutive days to a dose of 24 x and after that had rested for 5 days. *x*, abnormal mitosis of a fibroblast.

PLATE 20.

FIG. 4. Same block as in Fig. 1. Fibrinous exudate surrounding the foreign body.

FIG. 5. Inflammation of 42 days; fifteen exposures of 12 x each.

PLATE 21.

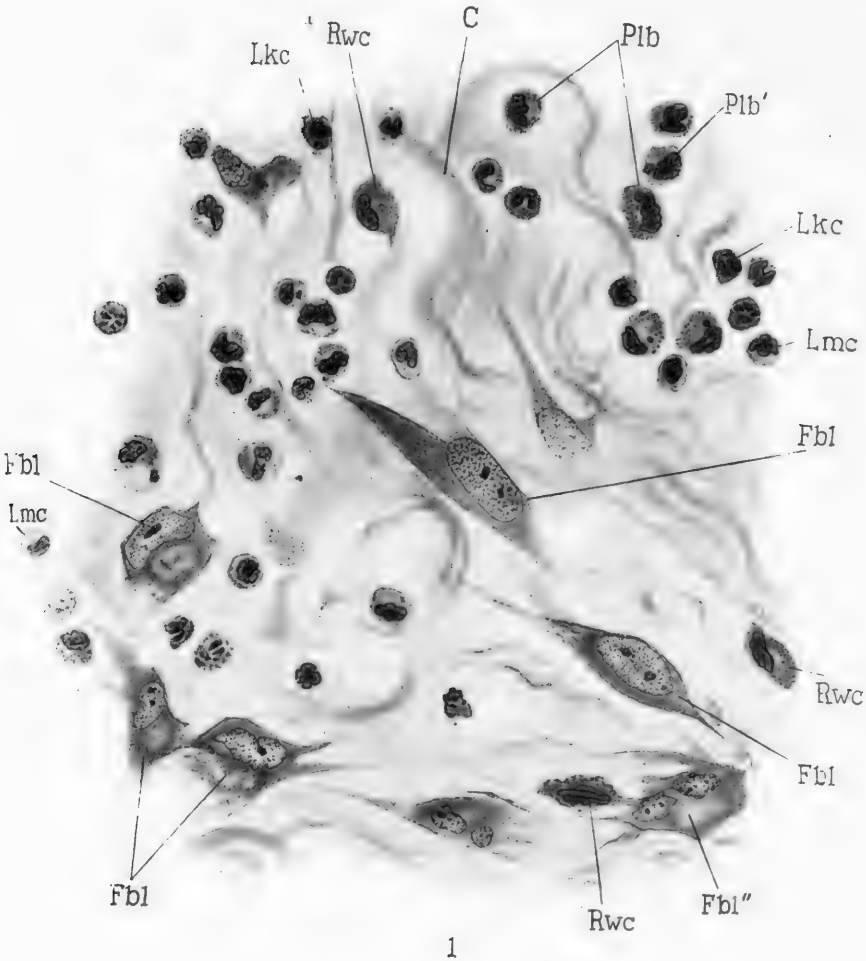
FIG. 6. Inflammation of 42 days; fifteen exposures of 12 x each.

FIG. 7. Same block as in Fig. 1. Oblique section of the wall of a small vein in the neighborhood of the exposed foreign body. Swelling and fragmentation of nuclei in endothelial cells.

PLATE 22.

FIG. 8. Inflammation of 23 days; fifteen exposures of 12 x each. Changes of the muscular fibers adjacent to the foreign body. *a*, *f*, *b*, *e*, different stages of degeneration of the fibers; *Am*, amitosis of a muscle nucleus.

FIG. 9. Same block as in Figs. 5 and 6. Later stages (*k*) of degeneration of the muscle fibers; *N*, groups of muscle nuclei.



1

(Maximow: Roentgen ray changes in inflamed tissue.)

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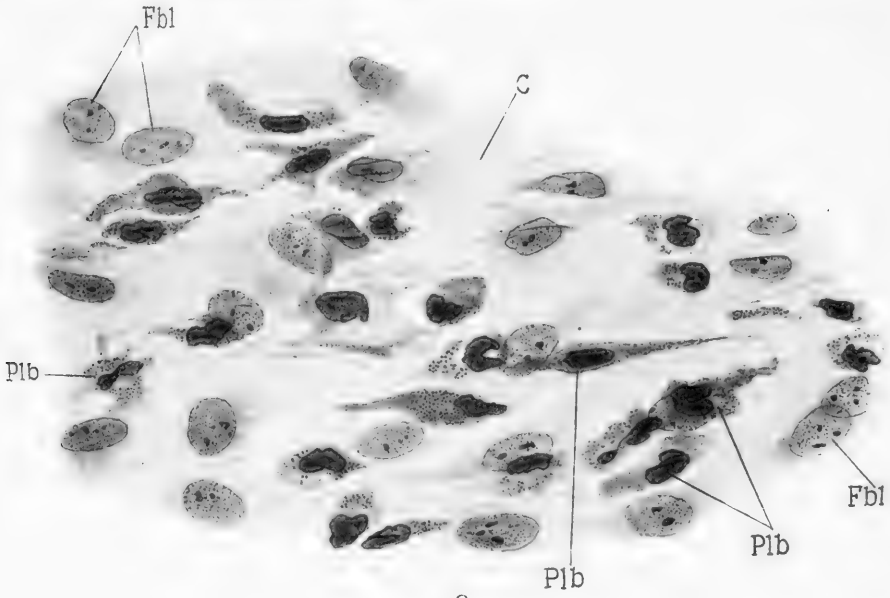

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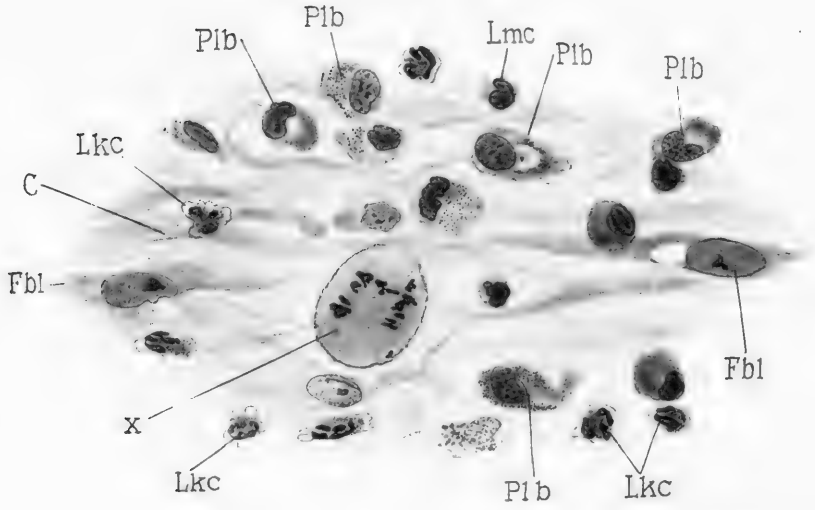

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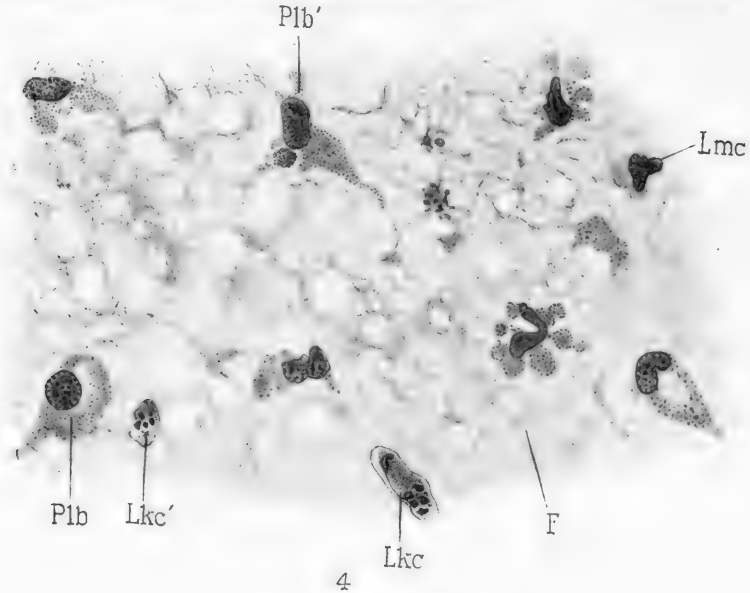

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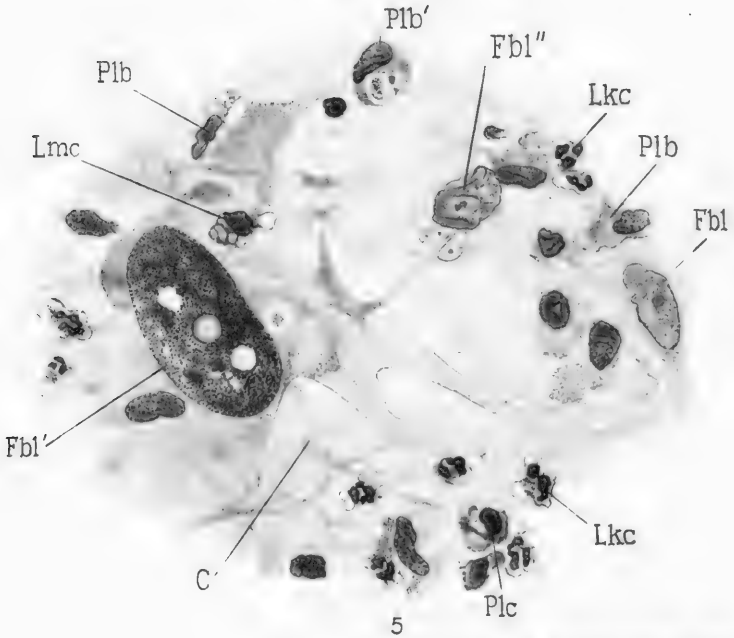

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(Maximow: Roentgen ray changes in inflamed tissue.)

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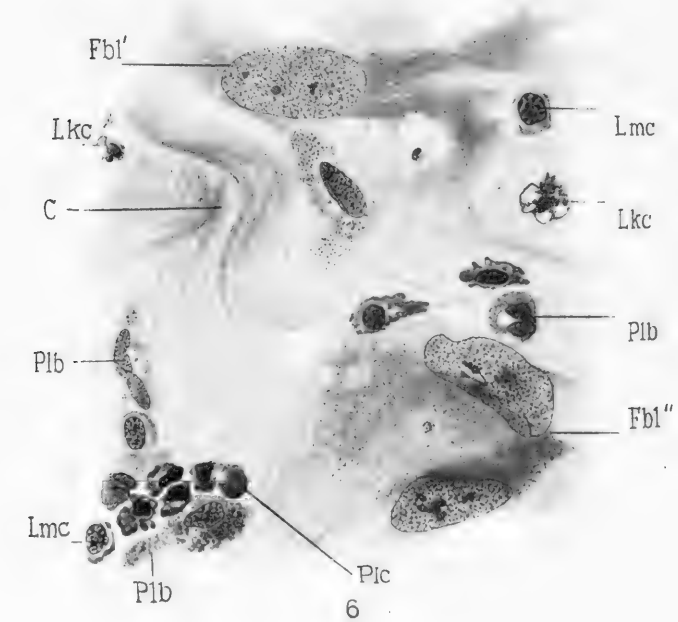

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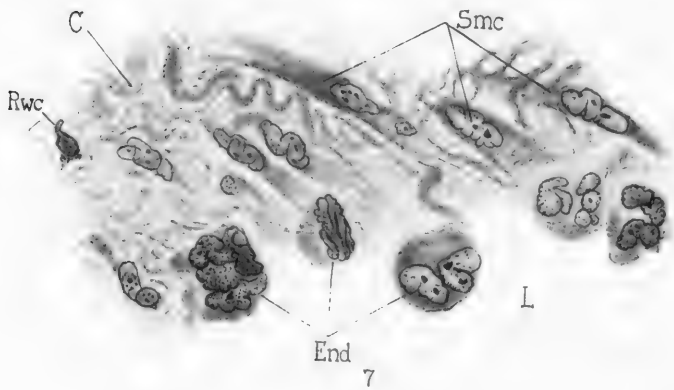

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(Maximow: Roentgen ray changes in inflamed tissue.)

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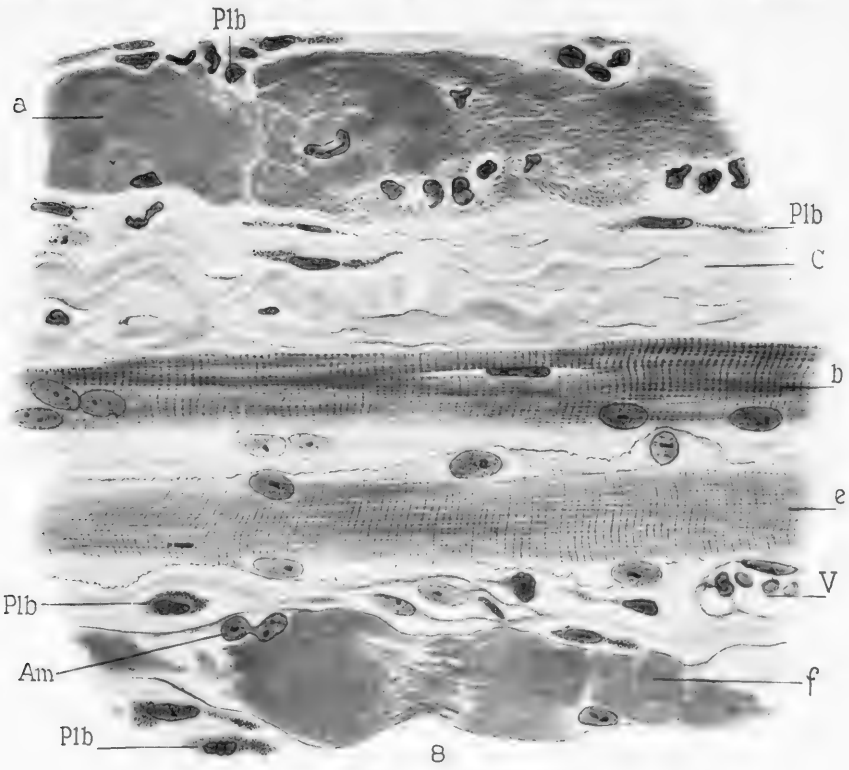

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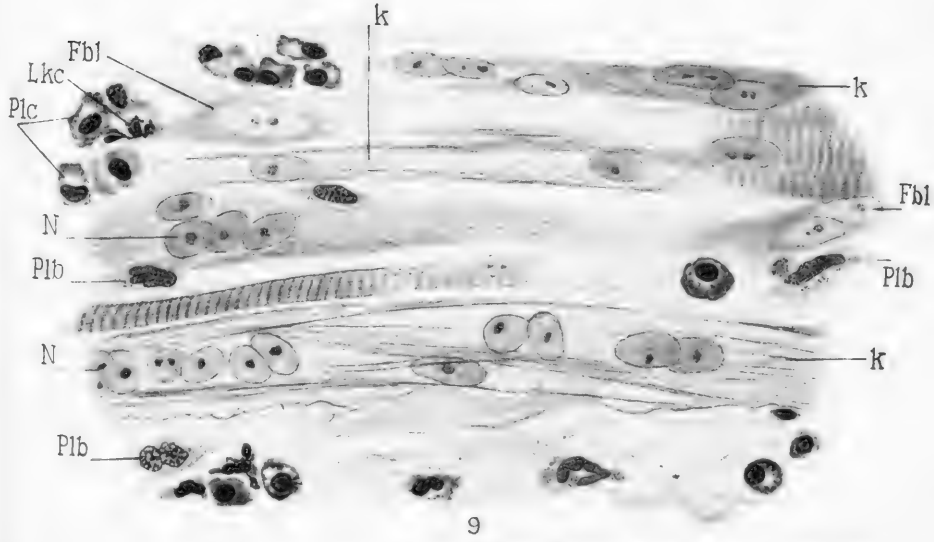

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(Maximow: Roentgen ray changes in inflamed tissue.)

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FURTHER STUDIES ON THE ETIOLOGICAL SIGNIFICANCE OF VIBRIO FETUS.

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PLATES 23 AND 24.

(Received for publication, November 6, 1922.)

To establish a new etiological entity it is necessary to study as many cases as possible of the spontaneous disease and to reproduce the lesions with pure cultures. The natural disease is the result of minimum doses of the microorganism having normal virulence. The artificially induced disease is the outcome of relatively large doses of a virus modified more or less by culture. The natural disease may not be reproducible but merely an approximate likeness of it. These facts must be kept in mind in estimating the value of evidence in etiological studies.

The importance of placental diseases in the cow warrants a continuation of somewhat detailed reports made upon vibrionic abortion since 1917.[1-4] In a former communication[4] the opinion was expressed that this disease might have been introduced from sheep and that the outbreak in which it was first identified had subsided. The inference that the disease had died out proved to be premature and observations made up to the present in the same herd seem to indicate that the disease is enzootic and probably a specific cattle disease of long standing to which the host species has become partially immune.

Since the first publication on vibrionic disease of the placenta several references have come to the writer's attention. Thomsen[5]

[1] Smith, T., *J. Exp. Med.*, 1918, xxviii, 701.

[2] Smith, T., and Taylor, M. S., *J. Exp. Med.*, 1919, xxx, 299.

[3] Smith, T., *J. Exp. Med.*, 1919, xxx, 313.

[4] Smith, T., Little, R. B., and Taylor, M. S., *J. Exp. Med.*, 1920, xxxii, 683.

[5] Thomsen, A., *Maanedsskr. Dyrlæger*, 1920, xxxii, 1.

working in Copenhagen was able to isolate spirilla from four of eleven cases of abortion. The four cases came from three different herds. Spirilla were found in stomach contents, tracheal mucus, and lungs of the fetuses and in fetal membranes and discharges. From two, pure cultures were obtained by the writer's method.

Gminder[6] is the first to have demonstrated vibrionic disease in Germany (Würtemburg). He observed two cases. There was nothing noteworthy about the fetuses. Placentas were evidently not obtained. Gminder produced abortion in pregnant guinea pigs with one to two loopfuls of culture injected subcutaneously. Inasmuch as the vibrios disappear from the peritoneal cavity of non-pregnant guinea pigs after injection of large numbers into that cavity within 2 or 3 days, the cause of abortion in Gminder's guinea pigs may be doubted. Lerche[7] also found a case of abortion associated with vibrios.

Spontaneous Cases.

Of spontaneous cases not hitherto reported, four were encountered in 1921 and five up to September, 1922. There is evidence that the cases are more numerous than this but the disease, being perhaps restricted to a small territory of the placenta, may fail to bring about premature expulsion of the fetus. It is scarcely necessary to go into great detail concerning these cases since this has been done in previous publications. The main fact to be emphasized in the present stage of our knowledge of this infectious process is the failure to detect *Bacillus abortus*.

Cow 621.—Received Nov. 20, 1920, from Michigan. Bred Dec. 3. Aborted June 12, 1921. On June 14, agglutination titer to *B. abortus* 1:40–.

A small portion of the placenta, hanging from the vulva, cut off for examination. About one-half of the chorion is beset with small yellowish excrescences. A small area is thickened and infiltrated with opaque, yellowish plaques. The cotyledons are pultaceous, yellowish. No normal ones in the portion obtained. Films show large and small rods, streptococci and vibrios. Some exudate cells contain them. In sections, small groups of villi are completely necrotic. Vibrios abundant in the edematous chorion.

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[6] Gminder, A., *Berl. tierärztl. Woch.*, 1922, xxxviii, 184.

[7] Lerche, *Deutsch. tierärztl. Woch.*, 1922, xxx, 281.

The fetus weighs 20 pounds. Without coat of hair. Nearly a liter of fluid in large serous cavities. Vibrios isolated from colon, rectum, liver, and kidneys. Cultures of other organs contain miscellaneous bacteria. Five guinea pigs inoculated with suspensions from uterine swab, contents of fourth stomach, rectum, and lung tissue of fetus failed to demonstrate *B. abortus*.

Cow 628.—Received Oct. 20, 1920, from Michigan. Bred Feb. 7, 1921. Aborted July 8. Placenta retained. On Sept. 29, agglutinin titer to *B. abortus* 1:20.

Fetus weighs 2,230 gm. Without coat of hair. Tissues edematous and partly macerated. Vibrios were isolated from lungs, rectum, kidney, and liver. Other tubes contain miscellaneous bacteria. Four guinea pigs inoculated with suspensions of lung tissue, contents of fourth stomach and rectum, and uterine swab were negative to *B. abortus* 10 weeks later.

Cow 643.—Purchased Oct. 12, 1918. Bred Feb. 27, 1921. Aborted, in third pregnancy since purchase, July 22. Placenta retained. Fetus weighs 2,410 gm. General edema of body. Meconium in stomachs.

Vibrios were isolated from fourth stomach and lungs. Five guinea pigs inoculated with suspension of uterine swab and with suspensions of fourth stomach, rectum contents, and lung tissue of fetus were negative to *B. abortus* 2½ months later.

This cow had been infected with *B. abortus* in earlier pregnancies as shown by the agglutinin titers. On June 22, blood 1:1,280, milk 1:80. July 23, blood 1:640–, milk 1:80. The interpretation to be placed on these data is that the cow had acquired enough resistance to *B. abortus* to prevent the latter from reaching the fetal membranes. The milk was not tested for *B. abortus*.

Cow 647.—Purchased Oct. 20, 1920, from Michigan. Bred Mar. 12, 1921. Aborted Aug. 25. Placenta retained. Agglutinin titer to *B. abortus*, Sept. 29, 1:20. Fetus 64 cm. long, without coat of hair. General edema and filling of abdominal cavity with blood-tinged fluid. Meconium in stomachs. Vibrios isolated from fourth stomach, rectum, liver, and lung. Four guinea pigs inoculated with suspension of uterine swab, contents of fourth stomach and rectum, and lung tissue of fetus were negative to *B. abortus* 2½ months later.

Cow 959.—Purchased May 22, 1919, from New York. Bred Jan. 3, 1921. Aborted June 15, 1922, in her fourth pregnancy since purchase. Agglutinin titer on day of abortion 1:40. Placenta retained. Fetus weighs about 4 kilos, and is 53 cm. long. No general edema or serous transudates. No evidence of autolytic changes. Contents of stomachs tinged yellow, otherwise normal. Slight edema of mesenteries. Films from contents of fourth stomach show vibrios of one to two wave-lengths. Large numbers of vibrios in contents of cecum. Two agar tubes plus lung tissue and fourth stomach contents and one tube from cecum and kidney tissue develop into pure cultures of vibrios. Tubes inoculated with spleen and liver tissue remain sterile. No other organism appears in the cultures.

Two guinea pigs which received into the peritoneal cavity a turbid suspension of fourth stomach and cecum contents mixed were killed 3 days after inoculation and cultures made by adding bits of spleen, liver, and kidney in the hope of recov-

ering the vibrio. Cultures all remain sterile. One guinea pig inoculated in the same way reserved to determine presence or absence of *B. abortus* was found negative after several months.

Cow 962.—Received Dec. 14, 1921, from Michigan. Bred Feb. 16, 1922. Aborted June 24. Placenta retained. Agglutination titer of blood 1:80, of milk 1:20–. Fetus about 35 cm. long, very limp, slightly discolored. Epidermis peels off in sheets. Abdomen torn open at umbilicus. Blood-tinged fluid partly filling pleural and peritoneal cavities. Epithelial layer of rumen comes away in a mass when organ is opened. Liver with markedly yellowish tinge. Placenta came away as a cylindrical tube, torn open at one end. The wall of tube markedly edematous, 5 to 6 mm. thick. Cotyledons in general pale yellowish, macerated. Films from cotyledons and contents of rumen show vibrios of about two wave-lengths. In this case a pure culture of vibrio was obtained from the contents of the rumen. The lung culture contained beside the vibrio colon bacilli. Two guinea pigs inoculated respectively with stomach fluid and a suspension of placental tissue in salt solution were found free from disease several months later.

Cow 965.—Received from Michigan in Oct., 1921. Aborted July 15, 1922. Placenta retained. Fetus is 41 cm. long and weighs 2,710 gm. General subcutaneous edema and effusion into large serous cavities. Vibrios were isolated from contents of fourth stomach and rectum and from spleen and liver tissue. Two guinea pigs inoculated with suspensions of uterine fluid obtained on a swab were normal when killed after 2 months.[8]

Experimental Production of Disease of the Placenta.

In the attempt to produce disease by inoculating animals with pure cultures of specific pathogenic bacteria, there are to be considered two determining factors. One is the original virulence of the strain or race as modified by culture conditions, the other the degree of specific natural resistance possessed by the host species. In endemic territories all degrees of disease may exist, some very slight and below the clinically manifest level. Thus cattle may receive intravenously doses of living *Bacillus abortus* and yet not contract placental disease, or if such disease starts it may be self-limited.

In a former communication[3] it was reported that of four cows inoculated with vibrios isolated from aborted fetuses, one had a

[8] Since this was written, two additional cases of vibrionic abortion have been studied. Cultures and guinea pig tests failed to bring *B. abortus* to light. The agglutinin titers of both cases towards *B. abortus* were low. The isolated vibrios were of the motile variety.

fairly extensive disease of the chorion, a second only slight lesions, and the remaining two none. No further attempts to reproduce vibrionic disease experimentally were made until 1922. The source of the virus was the placenta of a cow giving birth prematurely.

Cow 771.—Purchased in May, 1918. The present pregnancy is the fourth since purchase. Cow bred Aug. 17, 1921. Living calf born Mar. 31, 1922, therefore about 7½ months old. The calf died 7 hours after birth and was disposed of before it could be obtained for study.

About one-third to one-half of the placenta was obtained. The rest was adherent. The chorion was edematous but not otherwise visibly diseased. The cotyledons varied much in appearance. Several had portions of the maternal caruncle still attached. Some were quite normal. In several, yellowish gray areas within the cotyledon represented groups of degenerated villi. Some cotyledons had a fleshy, grayish aspect. Films prepared from the placental tissue present no bacteria recognizable as *B. abortus*. Among the few miscellaneous bacteria were vibrios of one to two wave-lengths. Within certain pale cells, probably necrotic polynuclears, were large numbers of very fine, degenerated vibrios (Fig. 1). In some cells the latter were well stained and of normal appearance. In sections of fixed and hardened tissue it was observed that the tissue under the chorionic epithelium was edematous and invaded by large groups of cells probably polynuclear. The blood vessels contain similar cells enmeshed in fibrin. Their walls are invaded by cells. On the surface, isolated, branched villi are denuded and densely filled with nuclear debris. Similar lesions occur here and there under the denuded surface. Epithelial cells wherever still *in situ* did not contain bacteria suggestive of *B. abortus* infection.

Since vibrios had been isolated once before by injecting suspensions containing them into the peritoneal cavity of guinea pigs,[2] an attempt was made to isolate the vibrio by injecting a turbid suspension of placental tissue in salt solution into the peritoneal cavity of three guinea pigs. They were chloroformed 3, 4, and 5 days respectively after inoculation. In those killed 3 and 4 days after inoculation, tubes of agar to which bits of spleen, liver, and kidney had been added and the tubes sealed developed pure cultures of a vibrio. All tubes inoculated in the same manner from the third guinea pig, killed after 5 days, remained sterile. At the same time two additional guinea pigs inoculated with the same suspension were kept slightly over 2 months and then killed. The animals were normal and the cultures sterile, thus indicating the absence of *Bacillus abortus*.

The twelve pure cultures obtained from the two guinea pigs served as the starting point for all subsequent experiments. The peculiarity of this new strain was an almost entire absence of motility (Figs. 7 and 8). An occasional active individual could be seen in hanging drop preparations in the original cultures from the guinea pigs. The bulk of growth was made up of long wavy forms, some nearly straight or only slightly curved or bent. The growth in all tubes was fairly vigorous and quite viscid. The vigor may be ascribed to the bits of guinea pig tissue, for it was barely visible in tubes of plain agar.

Stained films of this strain showed that the long wavy forms were composed of shorter forms, probably held together by some viscid capsular substance. Giemsa preparations brought out deeply stained coccus-like granules in the course of some of the long forms. Some granules were broader than the rods themselves[9] (Fig. 10).

A non-motile strain had been encountered once before[3] but no special study was made at the time beyond determining that it belonged serologically to the motile strains. Although the long forms made up the bulk of the cultures, yet in films from the placental tissue vibrios over two wave-lengths were not seen. We may therefore look upon the long forms as culture grouping of the non-motile variety.

Four cows, in different stages of pregnancy, were selected for inoculation to test the pathogenic character of the culture isolated through guinea pigs. Nos. 678 and 937 were inoculated with the same material. The feeble growth from five agar tubes plus cow's blood and two tubes plus bits of guinea pig spleen was suspended in bouillon. The cultures used were the third transfer, 7 days old. The inoculation was made both into the jugular vein and the subcutis, 7.5 cc. into the former and 3.5 cc. into the latter.

Cow 678.—Purchased in 1913. The fourth calf since purchase was born in May, 1921. Bred Aug. 28. Reacts to tuberculin. Titer to *B. abortus*, Mar., 1922, was 1:80. The injection was made as stated above at 3 p.m., Apr. 28. The temperature rose to 40°C. within an hour. It remained above 39°C. until early next day. After this it fluctuated slightly between 38.4° and 40°C. until May 5 when the temperature was taken only at irregular intervals and found

[9] See Smith,[1] Fig. 4.

slightly above normal. The cow was being "dried off" and milked either once a day or every other day. Milking was stopped after May 12.

A calf was born May 17, 7 p.m., 269 days after conception and 19 days after the injection of the vibrios. The placenta was adherent. Attempts by Dr. Little to remove it next day failed. To obtain the uterus with membranes before decomposition had gone too far the cow was slaughtered 20 hours after parturition, the uterus iced and brought to the laboratory. At the autopsy of the cow only the left bronchial lymph node was found tuberculous. When the uterus was opened with membranes attached the latter appeared normal and everywhere firmly attached to uterine caruncles. When the membranes were incised to expose the uterus and overlying chorion, the latter was found deeply injected and covered with a thin, soft, mucoid exudate gathered around the bases of the caruncles. When the cotyledons were pulled away from the caruncles, which required considerable force, the same kind of exudate was found resting on the villi. The pathological process was more clearly defined in sections of fixed and hardened tissue from various portions of the membranes. It was evidently quite different from that associated with *B. abortus*. In general it may be defined as a purulent exudation covering the chorion and penetrating into the cotyledons between fetal and maternal parts. It is probable that the uterine wall participated since its gland lumina were filled with polynuclear cell debris. Besides the exudate, individual villi and groups were densely filled with the nuclear debris of immigrated cells. Owing to the time elapsing between discharge of fetus and the killing of the cow, large bacilli and streptococci had invaded the placental tissues. However, vibrios were found in abundance in exudate from chorion and villi. Many cells contained masses of them (Fig. 2).

No direct cultures were attempted but guinea pigs were inoculated with the expectation that the vibrio might be isolated in this way. Two received into the peritoneal cavity suspensions in bouillon of material obtained from a uterine swab and two were inoculated with suspensions of diseased placenta previously ground in sand. Two guinea pigs were killed after 3 and two after 4 days. Twenty tubes of agar inoculated with bits of spleen, liver, and kidney remained sterile with one exception. This contained a plump rod. Two guinea pigs inoculated as above were reserved as tests for *B. abortus*. Both were found negative when killed after several months.

Cow 937.—Native cow. Calved in Jan., 1921. Bred Sept. 20, 1921. Reacted positively to tuberculin in Apr., 1922. Agglutinin titer towards *B. abortus* 1:80 in Dec., 1921, and 1:160 Apr. 22, 1922.

On Apr. 28, inoculated with and in the same manner as the preceding case (No. 678). A slight thickening of subcutis appeared at point of inoculation. The cow manifested no general disturbance, except a slight rise of temperature during the evening and night following the inoculation. The flow of milk having nearly ceased, the cow was milked only once a day from May 12 on.

On May 5, the lips of the vulva became swollen and edematous. Sacrosciatic ligaments were relaxed after May 11. On May 20, the cow dropped a premature

fetus at 5 a.m. after a period of gestation of 243 days. Fetus too weak to stand. Feet soft. Middle incisors not yet fully broken through. The others small and still covered with mucosa. Calf chloroformed after 4 hours and autopsied. Weight 41½ pounds. The organs were found normal with several exceptions. The mucosa of the entire leafy portion of the fourth stomach densely permeated with punctiform hemorrhages. Mucosa of small intestine markedly congested, especially in the middle portion. Lungs fully inflated.

Cultures were made from various organs. As might be expected, those of lung tissue, fourth stomach, and cecal contents developed a variety of large colonies due to invasion after birth. Tubes inoculated with bits of spleen, liver, kidney, urine, and heart's blood remained sterile.

The placenta remained adherent, excepting a small piece hanging from the vulva which was cut off for examination. Attempts to remove the placenta after 31 hours led to severe hemorrhage. The cow was therefore taken to an abattoir and killed at 5 p.m., May 21, 36 hours after the calf was born. The uterus containing the placenta was brought to the Institute's refrigerators soon after.

The placenta to be examined was the portion removed early and the remainder within the uterus obtained 1½ days after discharge of fetus. The detached piece contains seven large cotyledons. They are thick, hemorrhagic, with yellowish borders of a greasy, soft consistency, or else with yellowish islands throughout. The small cotyledons are completely softened and grayish in color. The chorion, uniformly injected, has on it small bits of mucoid exudate. Several small areas are infiltrated with 1 to 2 mm. smooth plaques. Films from different portions of this piece show large numbers of vibrios, single, and free (Fig. 4) and in cells (Fig. 3) in dense groups. No other bacteria detected.

On cutting open the uterus with attached placenta, there were found near the internal os a number of prominent caruncles which probably are those from which the piece of placenta had become detached.

The placenta still attached to the uterus presents for examination the chorionic, the amniotic, and the allantoic surfaces. The former is injected and here and there overlaid with yellowish flakes of exudate. The latter shows marked edema of the membrane overlying the cotyledons (allantois and amnion). When these are stripped off the cotyledons appear normal to the naked eye. When, however, the fetal portion of the cotyledons is pulled away from the maternal portion, groups of exposed villi are whitish among the normal red ones. Films from the uncovered cotyledons contain many vibrios.

The above conditions are largely explained in sections of fixed and hardened tissue. The detached villi are in part normal, in part densely filled with the nuclear debris of leucocytes denuded of epithelium. This is particularly true of the smaller villi and bases of the larger ones. Between the villi and resting on the chorion is a layer consisting of necrotic epithelium, masses of leucocytes, necrotic villi, and blood corpuscles.

On the cotyledons not separated from caruncles, smaller or larger areas of the section contain necrotic villi, evidenced by the fused, pink-staining cytoplasm

and pycnotic and fragmented nuclei of the epithelium. The same mixture of necrotic and leucocytic material is found layered between the surface of the maternal caruncle and the chorion facing it. Frequently this material consists chiefly of red blood corpuscles, forming a red streak between the maternal and fetal tissues.

The condition of the chorion varies from place to place. The epithelium may be still in place, or else gone and the tissue beneath densely filled with cell debris. Similar foci of necrotic leucocytes are found beneath the surface of the allantois and amnion. Small groups of villi forming the small adventitious cotyledons are as a rule necrotic. Vibrios are detected in some of these foci usually within cells. In certain portions of the chorion 36 hours old, foci of streptococci are prominent.

Cultures prepared from the deeper tissue of cotyledons, seared on the surface, contained both the long vibrios and streptococci (Fig. 11). The latter probably invaded the uterus immediately after discharge of fetus. No pure cultures were obtained. An attempt was made to isolate the vibrios through guinea pigs. Diseased placenta, ground with sterile sand and suspended in bouillon, was injected into the peritoneal cavity of three guinea pigs. One was reserved for a test for *B. abortus*. The two others were killed after 3 and 4 days respectively. Cultures made as in the preceding case (No. 678) remained sterile. The third guinea pig was negative to *B. abortus* infection several months later.

Two additional pregnant cows were inoculated with the same strain of vibrio. The growth from a number of agar cultures containing bits of guinea pig and calf tissues was brought together and suspended in bouillon. The inoculation was made into a jugular vein. The cultures were of the third transfer, and 8 days old.

Cow 712.—Native; grade Holstein. First calf born Aug. 27, 1921. Bred Nov. 19. Reacted to tuberculin. Agglutinin titer to *B. abortus*, Mar. 8, 1922, was 1:160+.

On May 2, injection of 7.5 cc. of a suspension of Vibrio 771 into a jugular vein. The cow showed a fall in the quantity of milk from 37 to 27 pounds the next day. On the day following the normal level had been reestablished. The temperature rose in 6 hours to 40.4°C. and gradually dropped to normal (38.4°C.) within 24 hours. Owing to the rapid invasion of the uterus after abortion by streptococci and anaerobic bacilli, it was decided to have the cow slaughtered and secure the uterus with fetus and membranes intact. This was carried out on June 13, 42 days after the injection of vibrios. The uterus was obtained about 1½ hours after the cow had been killed.

The lesion in this case was chiefly exudative and suppurative. In the central portion of the placenta, *i.e.* excluding the two horns, there is a heavy, viscid, pale grayish yellow exudate resting on the chorion and gathered chiefly at the bases of

the caruncles, extending from there up into the spaces between fetal and maternal portions of the cotyledons. Clustered about the cotyledons are numerous small adventitious groups of villi which are partly covered with exudate and have yellowish margins. The large cotyledons are normal to naked eye inspection. The cervical canal is tightly closed and the mucus plug about the external os of normal appearance.

Films of the exudate from various places on the chorion show the same characters. It is chiefly cellular, the cells being polynuclear plus the desquamated and necrotic epithelium. The consistency indicates the presence of much mucus. The exudate contains large numbers of vibrios, some nearly straight, long, slender, and simulating slightly bent filaments; the others wavy and much shorter, down to one wave-length in some cases (Fig. 6). Sections of fixed and hardened placenta confirmed what had been learned through naked eye inspection and stained films. There were general edema of chorion and villi, occasional necrotic villi, exudate between maternal and fetal portions of cotyledon, and diffuse cell infiltration of mucosa of uterus with occasional focal abscesses. The amniotic and allantoic fluids were of normal appearance. Cultures to which 0.5 to 1 cc. was added remained sterile.

The fetus is 31 inches long. The coat of hair is just appearing. The fluid of the stomachs is normal, the meconium dry. There are no abnormal conditions about the organs. Cultures prepared from contents of the fourth stomach and cecum and with bits of spleen and lung remained sterile. Cultures prepared from the exudate of the uterochorionic space developed into cultures of vibrios (Fig. 9). No other forms present. One culture contained many motile forms, the others only non-motile. Numerous tubes to which bits of tissue from the depth of cotyledons had been added remained sterile.

Three guinea pigs received into the peritoneal cavity 1 to 1.5 cc. of a turbid suspension of exudate, ground in sand and suspended in bouillon. One was reserved, the others were chloroformed after 2 and 3 days respectively. All cultures of the 3 day guinea pig remained sterile. Those from the guinea pig killed after 2 days contained spore-bearing bacilli and a diplococcus. The presence of these bacteria is due to the fact that the opened uterus had been reserved until the next day in the refrigerator before the inoculation was made. The third guinea pig was normal when killed several months later.

Cow 657.—Native. Received when a virgin heifer a dose of living *B. abortus*, Apr. 30, 1920. Gave birth to a normal calf Sept. 16, 1921. Placenta normal. At this time the agglutinin titer of the blood to *B. abortus* was 1:1,280, milk titer 6 days after parturition 1:640. Bred Oct. 17, 1921. Reacted to tuberculin Apr., 1922.

On May 2, 1922, when the fetus was 187 days old, the cow was injected with vibrios, at the same time and with the same dose as No. 712. The febrile disturbance was the same as with No. 712. Similarly, a drop in the milk secreted from about 24 to 17 pounds lasted but 1 day. The cow was slaughtered June 6,

35 days after inoculation, and the uterus with fetus secured for examination within 1½ hours. Agglutinin titer to *B. abortus* before death 1:320.

The lesions in this case were expressed by an exudate similar to that of No. 712, necrosis of cotyledons, and marked edema of the amnion covering them. The disease evidently started at one point in the occupied horn. Here a cotyledon is detached, greatly thickened, and largely transformed into a brownish, chocolate-colored, brittle mass. In it are immense numbers of vibrios. Adjoining this necrotic mass are several cotyledons covered with a thick, glass-clear, edematous layer, representing the amnion. These cotyledons, on section, show yellowish radial lines among the normal tissue. In addition to these changes, there are resting on the chorion small flakes of a smeary, greasy or mucoid character, grayish yellow in color. This exudate is concentrated around the bases of the maternal attachments of the cotyledons. Numerous vibrios are found in it (Fig. 5). Sections of the chorion and cotyledons show free necrotic villi filled with nuclear debris. The detached, necrotic cotyledon described above is shown with villi still within the maternal spaces. They are, however, filled with nuclear debris. The uterine wall is involved in its superficial zone which is infiltrated with cells having strongly basophilic cytoplasm. Some gland lumina and surrounding tissues are filled with polynuclear leucocytes. Fibrin lining the walls of the small chorionic vessels is occasionally seen. The sections show general edema of the chorion and attached villi.

The fetus was surrounded by a very turbid amniotic fluid, holding in suspension much flaky, yellowish material (meconium). Similar material deposited on the body. A cylindrical mass of soft meconium hanging from anus. The fetus measured 36 inches. The coat of hair was well developed, the teeth conspicuous. The stomachs contained a turbid fluid identical with the amniotic fluid. Similar fluid in trachea. The rectum contained very soft feces, the dry masses having been expelled. No edemas or transudations.

Cultures were prepared from the exudate on the chorion, the depths of the cotyledons, the organs of the fetus, and the fluid of the allantois. With few exceptions, all developed into pure cultures of vibrios. Thus of sixteen tubes inoculated either with exudate or tissue from cotyledons, *i.e.* still attached, seven contained the long, wavy, non-motile strain (Fig. 10) and one also motile forms, four remained sterile, and four contained one or more large contaminating colonies. Two tubes plus allantoic fluid contained motile vibrios only.

Cultures from the fetus resulted as follows: Two spleen cultures were contaminated, one tube plus liver tissue remained sterile. Pure cultures of vibrios were obtained from lungs, small intestine, rectum, and one liver tube. Active vibrios were present in all these tubes.

Four guinea pigs were inoculated, two with exudate and two with material from the necrotic cotyledon. One of each pair was reserved, the others killed after 3 days. From the latter pair all cultures remained sterile. The former were kept several months and then cultured with negative result.

DISCUSSION.

The evidence of the etiologic relation of the injected vibrios to the disease of the placenta in the four cows inoculated may be briefly summarized.

The vibrio used was isolated from a diseased placenta through two guinea pigs killed 3 and 4 days after inoculation. Pure cultures of a vibrio manifesting no motility were obtained from both guinea pigs (Figs. 7 and 8).[10] After three transfers, the cultures were injected intravenously in all four cows and subcutaneously in two of these. Two cows aborted. Two were killed and the fetal membranes with fetus obtained intact.

In all four the placenta was diseased. The process was characterized by edema of the fetal membranes, exudation in the uterochorionic space, and a fibrinous degeneration of the intima of the minute vessels. The exudate between chorion and uterine wall was made up of polynuclear cells, blood corpuscles, and debris from the epithelium of the chorion. Vibrios were detected in all cases in films. *Bacillus abortus* was not demonstrated either in sections, cultures, or through guinea pigs. The chorionic epithelium still present was free from bacteria.

Owing to the delay in obtaining the uterus with retained membranes from the two cows that aborted, only mixed cultures of vibrios and streptococci were obtained from No. 937. Cultures of vibrios were obtained in the case of the two killed cows from the exudate and cotyledons. The fetus of No. 712, 206 days old, was normal and its organs sterile. That of No. 657, 232 days old, had discharged large quantities of meconium into the amniotic fluid. Cultures of vibrios were obtained from the digestive tract and lungs.

The production of disease of the placenta had no clinical effect on the cows beyond a drop in the milk and a slight rise of temperature during the first 24 hours. So little disturbance was produced that the abortions came as a surprise. Nor was disease of the fetal membranes anticipated in the two cows killed.

[10] The protocols refer at first to the exceptional passage of a motile organism across the field of the microscope when cultures were studied in the hanging drop. Many later examinations failed to reveal such forms. The evidence as to the presence of motile individuals in this strain is therefore open to doubt.

Noteworthy is Case 657, a cow vaccinated before her first pregnancy with a living culture of *Bacillus abortus*. Her first calf was normal although her agglutination titer was high. Following inoculation with *Vibrio fetus* and consequent disease of the placenta, *Bacillus abortus* did not appear in any cultures or in the diseased tissues when these were subjected to microscopic examination.

The finding of motile vibrios in addition to the non-motile type demands attention at this point. The presence of motile forms in the culture injected remains in doubt, as stated above. Several interpretations of the data may be offered. (1) A very few motile forms may have been present in the original culture (No. 771) which owing to their motility penetrated farther and found their way into the fetus of No. 657. (2) The motile type may have been latent in one or more of the pregnant cows and under the depressing influence of the non-motile type gained access to the fetal membranes. Neither interpretation can be established since we know nothing definite of the whereabouts of these vibrios beyond their presence in the diseased membranes and the fetus. They are probably carried by some immune cows somewhere in the body. The two interpretations offered imply that the motile and non-motile forms are either mutants or else distinct races. Pending further study, little can be stated pro or con at the present time. The evidence seems to point to two distinct races of vibrios, a motile and a non-motile race. This inference is in accord with the fact that the disease due to the non-motile race attacks the amnion as well as the chorion. If this inference should prove true, the second interpretation, that the motile vibrio was latent in one or more inoculated cases, would stand. The possibility of such latency is given by the fact that the cows in the experiment came from the herd in which vibrionic disease occurs from time to time. The absence of reliable serological tests of infection or immunity makes it impossible absolutely to exclude infected or immune cows from experiments.

One additional step was taken to throw light on the significance of these strains.[11]

[11] The experiments given below were completed and some of the data added after the paper had been submitted for publication.

The non-motile strain (No. 771) isolated from the inoculated cow No. 657 and a motile strain isolated from a spontaneous case of vibrionic abortion were injected intravenously into two pregnant cows. The injections were made Oct. 23, 1922. The non-motile strain had been kept growing on agar plus a few drops of horse blood since early June. The motile strain had been isolated from Cow 959, also in June, and kept in the same manner. The dosage and method of injection were the same as in the former cases.

Cow 983 had been bred Apr. 26. The injection of the non-motile strain caused a slight febrile disturbance—the temperature going up to 40.1°C.—which subsided within 24 hours. The cow was killed Nov. 17, 25 days after the inoculation, when the fetus was presumably 205 days (6 months, 22 days) old. Both fetal membranes and fetus were normal. The injection had failed to cause disease.

Cow 982 had been bred Apr. 1. The injection of the motile strain caused the same temporary elevation of temperature as in Cow 983. The cow was killed Nov. 10, 18 days after the inoculation, when the fetus was 224 days (7 months, 10 days) old. In this case there was disease extending outward from the empty horn. The chorion was covered with patches of a viscid, mucoid, yellowish material, consisting largely of polynuclear leucocytes and an abundance of vibrios. The exudate was heaped up around the base of the caruncles. When the cotyledons were pulled away, slight peripheral disease of these structures appeared. There was slight edema of the amnion and allantois overlying the affected cotyledons. The fetus was 34 inches long. The only abnormality was a moderate amount of clear fluid in the abdominal cavity.

Agar tubes inoculated with the exudate on the chorion uniformly developed into pure cultures of the motile vibrio. This was furthermore isolated from the depths of a cotyledon, from the allantoic fluid, and from the lungs of the fetus. Cultures from other portions of the fetus and the amniotic fluid remained sterile.

The failure of the non-motile strain to produce lesions of the chorion in Cow 983 may be ascribed either to attenuation during the summer, or to a high resistance of the cow. The doubt that perhaps the non-motile strain was not instrumental in producing the lesions of the early four cases, in view of the presence of the motile strain, is dispelled by the fact that the non-motile strain was isolated in a number of tubes in pure culture from the chorionic exudate of both earlier cases killed before abortion. In No. 657, none of the exudate tubes contained motile forms. In No. 712, only one out of a number contained them.

CONCLUSION.

The general outcome of these six experimental cases and those already reported[3] is that both motile and non-motile types produce disease of the fetal membranes eventually leading to abortion. The more precise serological relations between the two are now under investigation.

EXPLANATION OF PLATES.

Figs. 1 to 6 are photomicrographs of films made directly from the diseased placenta. Figs. 7 to 11 represent cultures. In all, the non-motile, only slightly wavy type of vibrio is present. Figs. 1, 7, and 8 represent the strain of vibrio used for injection. All the rest are from the injected animals. Magnification, × 1,000. For figures of the motile type the reader is referred to a former publication.[1]

PLATE 23.

FIG. 1. Film prepared from the placental exudate of Cow 771, the source of the vibrio used in the experiments. Note the cell, probably a polynuclear leucocyte, with irregular basophil masses on the periphery, which are chromatin remains of the nucleus. The space between them represents cytoplasm. This contains a number of vibrios and point-like bodies, some of which are vibrios in optical section, others of unknown nature. Three vibrios are free above the cell. Giemsa stain.

FIG. 2. Film from the placenta of Cow 678, injected with a culture of vibrio. Note several free vibrios and a necrotic cell containing a number of organisms, some distinctly wavy, others simply curved. The deeply stained mass is a bit of placental tissue.

FIG. 3. Film prepared from beneath the seared surface of a cotyledon of Cow 937. It shows two cells densely stuffed with vibrios. The nuclear matter appears as clumps on the cell periphery. The dark mass is a bit of placental tissue.

FIG. 4. Film prepared from the placenta of Cow 937. Note the vibrios of one to several very shallow turns or wave-lengths among the cellular debris of the exudate. Stained in Giemsa's solution 24 hours.

FIG. 5. Film from the exudate on the chorion of Cow 657. Many organisms are among the cell masses, some wavy, others nearly straight.

FIG. 6. Film from the viscid exudate, resting on the chorion, of Cow 712. Many vibrios are present, closely resembling those of Fig. 5.

PLATE 24.

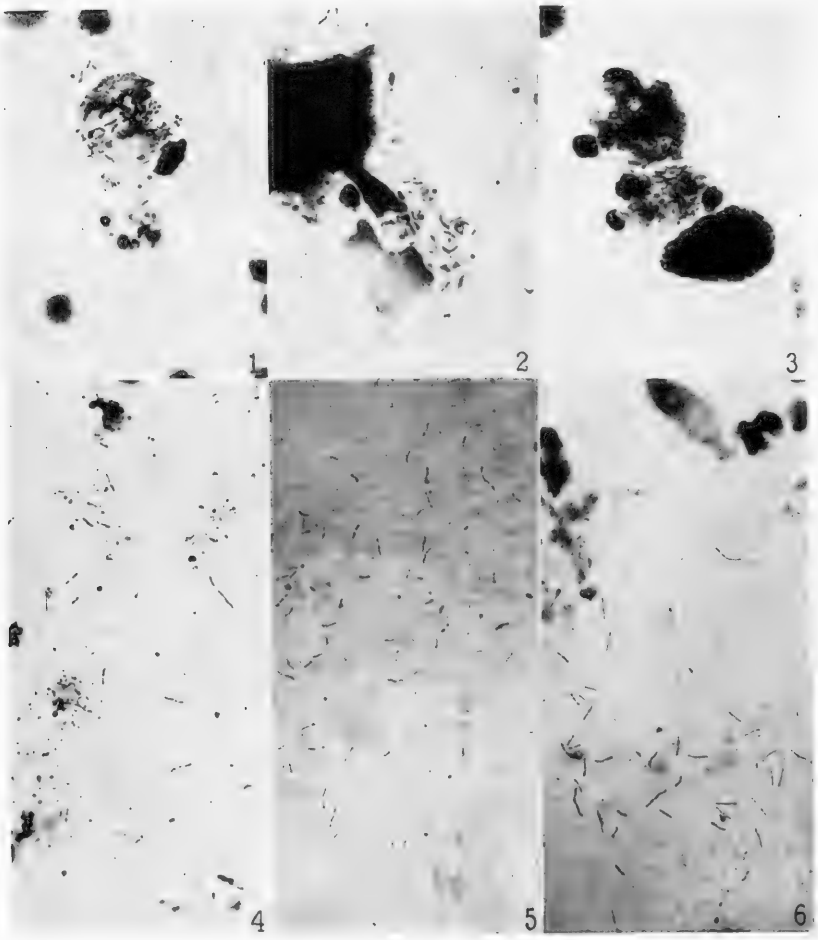
FIGS. 7 and 8. Films prepared at the same time from a culture of the vibrio from Cow 771, isolated through a guinea pig. This is the sixth transfer, 6 days

old, on slanted agar containing a bit of calf kidney in the condensation water. The thickness of the stained vibrios can be modified by changing the amount of the Giemsa stain added to distilled water. In Fig. 7 the stain was sufficiently concentrated to make the vibrios appear unusually large as compared with those of Fig. 8 from the same culture.

FIG. 9. Culture from a bit of exudate from the chorion of Cow 712, on slant agar, sealed, 3 days old. Note both long and short forms of distinct vibrio form. The longer the vibrios, the less marked the spiral or wavy form. Alkaline methylene blue.

FIG. 10. Culture on plain agar plus some exudate from the chorion of Cow 657. There are present very short and very long forms, all distinctly wavy. The deeply stained round bodies of varying size are probably free, overstained granules which are found in long spirals of old cultures. Film stained with Giemsa's solution.

FIG. 11. Mixed cultures of vibrios and streptococci from a cotyledon of Cow 937, to show the relative size and staining capacity of the two species.



(Smith: Etiological significance of *Vibrio fetus*.)

(Smith: Etiological significance of *Vibrio fetus*.)

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THE INCREASED ABSORPTION OF X-RAYS BY VITALLY STAINED WHITE RATS.*†

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In a previous paper a series of experiments was reported demonstrating the action of x-ray energy upon paramecia which had been stained with certain vital stains; namely, Nile blue sulfate, alizarine blue S, trypan blue, isamine blue, Nile blue hydrochloride, trypan red, dahlia (Grübler), neutral red, aniline red, Sudan III (oil), Janus green (Eimer and Amend), and methylene blue (BX Merck).[1] It was ascertained by this study that a dosage of from 50 to 60 milliampere minutes[2] produced death within 48 hours in those organisms which had not been subjected to the stain. On the other hand, the presence of the stain within the cytoplasm of the paramecia so greatly increased the absorption of x-ray energy that a dosage of from 5 to 10 milliampere minutes given under the same conditions of x-raying was sufficient to produce death within 48 hours. The most plausible explanation of this phenomenon which could be presented at the time was that the permeability of the nuclear membrane to the stain had altered. This alteration was dependent, apparently, upon two factors, the presence of the stain in immediate proximity to the nuclear membrane, and the action of the x-ray energy.

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† Financial assistance for this study has been given by the Rumford Committee of the American Academy of Arts and Sciences, and through the courtesy of the General Electric Company at Schenectady the work has been made possible.

[1] Baldwin, W. M., A study of the combined action of x-rays and of vital stains upon paramecia, *Biol. Bull.*, 1920, xxxix, 59.

[2] The amount of radiation is calculated as the product of the current strength in milliamperes and the time of exposure in minutes and is expressed as milliampere minutes.

It was difficult to stain the nucleus in the paramecium while the organism was alive. Diffusion of the stain into the nucleus indicated ordinarily the ensuing death of the organism. It was found, on the other hand, in most marked contrast to this condition that the carrying capacity of the cytoplasm for the stain was relatively great. A careful examination of the experimental data demonstrated an increased absorptive property of the nucleus for the stain which resulted invariably in the death of the paramecium.

Since the publication of these results investigation has been prosecuted along the same line, of the chemical sensitization of tissue cells to x-ray energy, by the use of adult white rats. While a wide variation has been noted in the behavior of adult mammalian tissues to the combined action of vital stains and x-rays, the general principles underlying the protoplasmic reactions to this combination of experimental conditions may be illustrated by the results obtained with trypan blue. No effort was made to test out the minimum lethal dose of this stain for rats.

Method.

The dry stain was dissolved in the proportion of 0.25 gm. to 50 cc. of sterile 0.6 per cent sodium chloride solution. Each rat received a hypodermic, subcutaneous injection of from 2 to 4 cc. of stain, depending upon weight, on the morning of the 1st, 3rd, and 5th days. On the afternoon of the 5th day, after the injections of stain had been completed, a dosage of 100 milliampere minutes was given at a distance of 17.5 cm. from the target. The source of the x-ray energy was a large, water-cooled, Coolidge tube, the surface of which was additionally cooled by a strong air blast. A thin layer of pasteboard separated the rats from the target. The compartment of the container holding the rats was adjustable to the extent that once in position the animals could not move about. Consequently, the maximum dose of x-ray energy was received by the skin upon the side of the rat turned toward the tube.

EXPERIMENTAL.

A significant feature of the paramecium experiments obtained likewise with the stained rats. With the former, the absorption capacity for x-ray energy varied within wide limits, depending upon the different strains of paramecia. Similarly, the stained white rats exhibited a wide range of susceptibility. It may be stated in gen-

eral, however, that a dosage of $\frac{100 \text{ MAM}}{17.5 \text{ cm.}^2}$ at 50 kilovolts of x-ray energy induced death in the unstained rats within from 110 to 120 hours. On the other hand, the same amount of energy, acting under the same experimental conditions upon rats which had been vitally stained, shortened the period of viability to from 60 to 70 hours. This fact may be interpreted as indicating, therefore, an increase of almost 100 per cent in the susceptibility of the stained over the unstained rats to this form of energy. Some data upon the point will be given.

On Dec. 2, 1916, at 11 a.m., six adult white rats were x-rayed. Two were given a dosage of $\frac{75 \text{ MAM}}{17.5^2}$ at 50 kilovolts, two a dosage of 50 milliampere minutes, and two a dosage of 25 milliampere minutes, all under the same conditions. On Dec. 6, one rat which had received a dose of 75 milliampere minutes died, 90 hours after the initial x-raying. On Dec. 10, one rat which had received 75 milliampere minutes died, 195 hours after x-raying. The observation was made on Dec. 30 that both of the rats that had received 50 milliampere minutes and both that had received 25 milliampere minutes were alive and well. One adult white rat was x-rayed on May 1, 1917, at 10.30 a.m., with a dosage of $\frac{100 \text{ MAM}}{17.5^2}$ at 50 kilovolts. This rat died on May 5, 97 hours after the x-raying.

Another experiment was conducted on Sept. 8, 1916. Two adult white rats were x-rayed at 2.15 p.m. They were given a dosage of $\frac{125 \text{ MAM}}{22.5^2}$ at 50 kilovolts. One died on Sept 14, 115 hours after x-raying; the other died on Sept. 15, 139 hours after x-raying. Furthermore, on Sept. 8, two adult white rats were given a dosage of $\frac{500 \text{ MAM}}{22.5^2}$ at 50 kilovolts, and two a dosage of $\frac{250 \text{ MAM}}{22.5^2}$ at 50 kilovolts. The rats which were given the larger dosage of 500 milliampere minutes died on Sept. 12, 80 hours after x-raying; the two which had been given 250 died on Sept. 12, 84 hours after x-raying.

Another experiment was conducted on July 11, 1916, at 1.30 p.m. Four rats were x-rayed under the same conditions, with a dosage of $\frac{50 \text{ MAM}}{17.5^2}$ at 50 kilovolts. One died on July 14, 96 hours after x-raying; three died on July 15, 108 hours after x-raying.

It was ascertained from experiments similar to the above that the period of viability of the average adult white rat, after a dosage of x-ray energy of $\frac{100 \text{ MAM}}{17.5^2}$ at 50 kilovolts, varied between 110 and 120

hours. A greater dosage, as has been seen in the instance of 125, 250, and 500 milliampere minutes, shortened this period. This standard dosage of 100 milliampere minutes was selected for the purpose of the experiment as what might be termed provisionally the minimal lethal dosage for the period.

This period of viability of x-rayed rats was considerably shortened, however, when injections of trypan blue in aqueous solution were administered to the rats in dosages varying between 2 and 4 cc. of a 0.5 per cent solution. This will be seen by the following extracts from the experimental notes.

Two rats were x-rayed on Sept. 28, 1916, at 2.15 p.m., at $\frac{100 \text{ MAM}}{17.5^2}$ at 50 kilovolts. One rat died on Sept. 30, 60 hours after x-raying, and the other on Sept. 31, 70 hours after x-raying. On Oct. 2, one rat was x-rayed at 5 p.m., under the same experimental conditions, and died 73 hours later. Still another rat was x-rayed on Oct. 3, at 11.30 a.m., and died on Oct. 5, 40 hours after x-raying. On Jan. 9, 1917, one rat was x-rayed at 9.45 a.m., after the standard injections of trypan blue had been administered. This rat died 75 hours after x-raying. On Apr. 24, one rat was x-rayed at 10.15 a.m., at $\frac{100 \text{ MAM}}{17.5^2}$ at 50 kilovolts after two injections of trypan blue had been administered, 1 day intervening between the injections. This rat died on Apr. 27, 70 hours after x-raying. On Apr. 25, one rat was x-rayed, under the same conditions, after three injections of trypan blue had been administered, 1 day likewise intervening between injections. This rat died on Apr. 28, 67 hours after x-raying. On Apr. 27, another was x-rayed under the same conditions, after two injections of trypan blue, 1 day intervening between injections. This rat died on Apr. 29, 60 hours after x-raying.

These are merely typical examples of the results encountered with more than 50 stained rats utilized for the purpose of the investigation. The extreme experimental variations are well illustrated by the few examples given. The average results indicate, however, a marked increase in the susceptibility of the rats to the lethal effects of x-ray energy in the presence of the stain.

The results of x-ray experiments conducted upon developing frogs' eggs have been previously reported.[3] In these experiments it was

[3] Baldwin, W. M., The artificial production of monsters conforming to a definite type by means of x-rays, *Anat. Rec.*, 1919–20, xvii, 135; The artificial production of monsters demonstrating localized defects as the result of injury from x-rays, 1920–21, lii, 296; Effects produced by x-ray energy acting upon frogs' ova at early developmental stages, *Science*, 1920, lii, 229.

noted that the developing brain and spinal cord, the epidermis, the enteron, the heart, and the vascular system in general showed a greater specific susceptibility to the destructive action of x-ray energy than most of the other tissues of the embryo. Extended studies gave warrant for the hypothesis that the mitotic activity of the cells in embryonic tissues was in direct ratio to the susceptibility of these cells to x-ray energy. This, in a way, conforms to the general principle previously discovered by other investigators that during the mitotic period the susceptibility of the cell to injury from various forms of physical energy is increased. Moreover, as a direct corollary to this, it was found that the absorptive capacity of tissues most rapidly differentiating greatly exceeded that of the less active tissues. The working hypothesis is not without support that, apart from chemical constitution, the determining factor in specific susceptibility of tissues is very largely, if not entirely, mitotic activity.

The cytoplasmic and nuclear detritus gave evidence that all types of embryonic cells absorbed a certain amount of energy. This contrasted markedly with the relative insusceptibility of these same cell types in adult tissues. Repeated experimentation verified the fact that in the adult the sex glands, bone marrow, leucocytes, spleen, and the epidermis on the side of the body turned towards the tube were most readily affected by x-ray energy. On the other hand, cells of what may be called mitotically inactive types of adult tissues, such as brain and spinal cord, muscle, glandular, and connective tissues, were relatively insusceptible or lacked an absorptive capacity for this form of energy. These findings support the working hypothesis derived from a study of embryonic tissues.

It should be stated that this working hypothesis must conform with the well established physical principle underlying x-ray absorption—the higher the atomic weight of the element, the greater the proportion of x-ray energy absorbed—and, in addition, must allow for those active, soft rays which are almost entirely absorbed by the skin on the side turned towards the tube.

In view of the results with embryos noted above, it was somewhat disappointing that the vitally stained rats which had been x-rayed revealed no departure from the histologic picture presented by the x-rayed but unstained rats. The more actively mitotic tissues,

such as the spleen, the bone marrow, and the sex glands, were deeply stained, and showed wide cytologic variations from the normal both in the number and in the kind of their cellular elements. A considerable proportion of the stain had been taken up by the nuclei of the cells of these organs and of the connective tissues as well.

As in embryos, when the quantity of the x-ray energy was sufficiently great, its injurious action upon both nucleus and cytoplasm was manifested through the presence of both nuclear and cytoplasmic extrusions. In the embryo, all of the body cavities, especially the neurocele, contained relatively large amounts of this detritus, the quantity standing in direct ratio to the amount of energy utilized. It stood in direct proportion, moreover, to the destructive effects upon the cells lining these cavities. With this phenomenon as an indicator, through careful histologic technique it was possible to recognize the same evidences of x-ray energy in the more actively mitotic tissues of the adult rat. Further evidence, belonging in the same category, is to be found in the increase noted in the nitrogen output and uric acid elimination, and in the increased elimination of purine bases and of P_2O_5. It might be inferred that all these effects, which have been observed by others, as well as the author, and are most noticeable in the excretory organs of the body, collectively point to the assumption that such pathologic changes are secondary to, and not the primary effects of, the injurious action of x-ray energy upon cytologic constituents and cytologic metabolism. In both the stained and the unstained rats, the necrotic foci in the mucosa of the small intestine and in the convoluted tubules of the kidney, and the areas of leucocytic infiltration in the liver, observed by Hall and Whipple[4] and by Warren and Whipple,[5] are to be thought of as secondary, not primary, effects of x-ray energy.

[4] Hall, C. C., and Whipple, G. H., Roentgen-ray intoxication: Disturbances in metabolism produced by deep massive doses of the hard roentgen rays, *Am. J. Med. Sc.*, 1919, clvii, 453.

[5] Warren, S. L., and Whipple, G. H., Roentgen ray intoxication. I. Unit dose over thorax negative—over abdomen lethal. Epithelium of small intestine sensitive to x-rays, *J. Exp. Med.*, 1922, xxxv, 187; II. A study of the sequence of clinical, anatomical, and histological changes following a unit dose of x-rays, 203; III. Speed of autolysis of various body tissues after lethal x-ray exposures. The remarkable disturbance in the epithelium of the small intestine, 213.

The assumption by tissue cells of a spherical form and the staining of the nuclei have been considered in this investigation as indicative of the injurious effects of the energy, in accordance with the observations on the embryo. These changes were most marked in the bone marrow, spleen, and sex glands. Towards the end of the life of the x-rayed rat such injured cells almost completely degenerated or disappeared.

DISCUSSION.

The peculiar sensitizing action of the stain as described is comparable to that of an optical sensitizer such as eosin, and in all probability results from the adsorption of the stain on the surface of the nucleus, a physical phenomenon which occurs most actively under the direct influence of an electromagnetic form of energy. Evidence for such a phenomenon was given first by Hertel[6] who, working with the effect of ultra-violet light ray energy upon bacteria in 1905, found that a certain wave-length not injurious to the bacteria was absorbed by them in the presence of eosin with consequent injurious effect, providing the eosin possessed an absorption band within the range of wave-length utilized. Furthermore, the colloidal selenium work of Henri[7] bears out the same theory. The sensitizing effect depends then apparently upon two separate and distinct factors: first, the intimate

[6] Coehn, A., and Barratt, J. O. W., Ueber Galvanotaxis vom Standpunkte der physikalischen Chemie, *Z. allg. Physiol.*, 1905, v, 1. Barratt, J. O. W., Die Addition von Säuren und Alkalien durch lebendes Protoplasma, *Z. allg. Physiol.*, 1905, v, 10. Nagai, H., Erstickung und Narkose des Flimmerepithels, *Z. allg. Physiol.*, 1905, v, 34. Hertel, E., Ueber physiologische Wirkung von Strahlen verschiedener Wellenlänge. Vergleichend-physiologische Untersuchungen. II. Mitteilung, *Z. allg. Physiol.*, 1905, v, 95.

[7] Henri, V., and Wurmser R., Etude de la loi d'absorption photochimique pour les réactions produits par les rayons ultraviolets, *Compt. rend. Acad.*, 1912, clv, 503. Henri, Mme. V., Henri, V., and Wurmser, R., Etude quantitative de l'absorption des rayons ultra-violets par l'albumine d'oeuf et le sérum, *Compt. rend. Soc. biol.*, 1912, lxxiii, 319. Henri, V., Comparaison de l'action des rayons ultra-violets sur les organismes avec les réactions photochimiques simples et complexes, *Compt. rend. Soc. biol.*, 1912, lxxiii, 323. Henri, V., and des Bancels, J. L., L'excitation provoquée par les rayons ultra-violets comparée avec les excitations visuelles et nerveuses, d'une part, et les réactions photochimiques de l'autre. Lois des phénomènes, *Compt. rend. Soc. biol.*, 1912, lxxiii, 328.

association of the foreign chemical with the living protoplasm of the cell; and, secondly, the presence of a form of physical energy. The following evidence must be considered before a rejection of this theory can be entertained. Solutions of the stain that had been subjected to a relatively enormous dosage of x-ray energy produced no lethal effect when afterwards injected into the rats; the period of viability of x-rayed rats was not shortened when they were subsequently injected with the stain. No change in the appearance of the stain or in its distribution within the cytoplasm was demonstrable as the direct result of radiation. Prolonged radiation did not alter the color absorption band or other physical properties of the stain either in the powdered form or in solution.

SUMMARY.

The object of this investigation was to test out the sensitizing effect of various vital stains upon rat tissues in the presence of x-ray energy. For the purposes of the experiment, it had been found that a dosage of $\frac{100 \text{ MAM}}{17.5 \text{ cm.}^2}$ at 50 kilovolts induced death in white rats within 110 to 120 hours. This amount of energy was selected as a tentative standard for the experiments. The preliminary injection of small amounts of such stains as trypan blue in 0.5 per cent aqueous solution sensitized the rat tissues to such an extent that the period of viability of these rats after administration of the standard dosage of x-ray energy, was shortened to from 60 to 70 hours. The cause of death in the stained rats has not been ascertained despite careful histologic study. Considerable variation in the susceptibility of rats to the combined procedure was noted.

CHEMICAL CHANGES IN THE BLOOD OF THE DOG AFTER INTESTINAL OBSTRUCTION.

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(Received for publication, October 19, 1922.)

In a study of three patients who developed acute symptoms following gastroenterostomy, it was shown that the important chemical changes were an increase in the non-protein nitrogen of the blood coinciding with a very high nitrogen excretion in the urine, an increase in the carbon dioxide-combining power of the blood plasma, a marked fall in chlorides in the whole blood, and an almost total absence of chlorides in the urine.[1] The symptoms and the changes in the chemistry of the blood we have interpreted as resulting from intoxication brought about probably by an obstruction of the duodenum at the gastroenterostomy stoma. These findings have led us to investigate more completely the chemistry of the blood of dogs with intestinal obstruction to ascertain whether changes such as were observed clinically occur when the intestine is experimentally obstructed.

It is a generally accepted fact that the symptoms in intestinal obstruction are due to the action of some powerful toxic agent which develops soon after the obstruction occurs. It has been abundantly proven also that the toxic agent destroys protein at a rapid rate. This is shown by the great increase in nitrogen excretion, which is coincident usually with an increase in the non-protein nitrogen of the blood.

Tileston and Comfort[2] were the first to point out the increase in blood nitrogen. Later Cooke, Rodenbaugh, and Whipple[3] studied experimentally the chemical changes in dogs following intestinal obstruction. They confirmed the findings reported by the first two observers, that there is a gradual increase in non-protein

[1] Haden, R. L., and Orr, T. G., *Bull. Johns Hopkins Hosp.*, 1923, xxxiv, 26.

[2] Tileston, W., and Comfort, C. W., Jr., *Arch. Int. Med.*, 1914, xiv, 620.

[3] Cooke, J. V., Rodenbaugh, F. H., and Whipple, G. H., *J. Exp. Med.*, 1916, xxiii, 717.

nitrogen and urea nitrogen. They also made single studies on eight dogs and one man of the uric acid, creatinine, creatine, and amino-acid nitrogen, in addition to the total non-protein nitrogen and the urea nitrogen. Their conclusions were that there is always an increase in the non-protein nitrogen, that the urea nitrogen is not a good index of the rise in total blood nitrogen, and that the other non-protein nitrogenous bodies show no significant change except an occasional rise in creatinine. Draper[4] reports a chemical study of the blood in one dog, with essentially the same findings, except that the uric acid was high before death. Louria[5] and Rabinowitch[6] have reported findings in human cases of obstruction similar to those in experimental animals and they have emphasized the value of the study of the blood nitrogen in the differential diagnosis and prognosis of acute abdominal conditions.

There have been few studies reported on the bicarbonate content of the plasma in intestinal obstruction. Whitney makes the statement[7] that there is no acidosis. Draper found in one dog a gradual fall in the alkali reserve. Odaira[8] has studied the reaction of the blood following intestinal obstruction in rabbits, using the hemoglobin dissociation constant method, and concludes that there is always an acidosis which is relieved by the administration of sodium bicarbonate. We can find no record of studies on the blood chlorides in the literature.

Method.

Dogs were used throughout the experiments. All operations were done under ether anesthesia with aseptic technique. The animals were kept in metabolism cages and were given no food after operation and usually none for 48 hours preceding operation. They were allowed water *ad libitum*. Blood for chemical analysis was obtained from the jugular vein before operation and at 24 hour intervals thereafter until death.

The non-protein nitrogen, sugar, and creatinine were determined by the method of Folin and Wu,[9] amino-acid nitrogen by the method of Folin,[10] the carbon dioxide-combining power by the method of Van Slyke,[11] urea nitrogen by the Van Slyke and Cullen

[4] Draper, J. W., *J. Am. Med. Assn.*, 1917, lxix, 1768.
[5] Louria, H. W., *Arch. Int. Med.*, 1921, xxvii, 620.
[6] Rabinowitch, I. M., *Canad. Med. Assn. J.*, 1921, xi, 163.
[7] Whitney, J. L., *Arch. Int. Med.*, 1917, xx, 931.
[8] Odaira, T., *Tohuku J. Exp. Med.*, 1921, ii, 570.
[9] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.
[10] Folin, O., *J. Biol. Chem.*, 1922, li, 377.
[11] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.

modification[12] of the Marshall method, and uric acid by the method of Benedict.[13] The chlorides were determined on the tungstic acid filtrate in the manner suggested by Gettler.[14] For the non-protein nitrogen in the urine Folin's micro method[15] was used, and for the chlorides a modified Volhard-Arnold method.

OBSERVATIONS.

The chemical findings in the blood of twenty-five normal dogs have been summarized in Table I to serve as a basis in interpreting the results obtained in the experimental animals. The average non-protein nitrogen is 30.3 mg., urea nitrogen 11.1 mg., uric acid 1.5 mg., creatinine 1.5 mg., amino-acid nitrogen 6.3 mg., sugar 76 mg., and chlorides 458 mg. per 100 cc. of blood. The average CO_2-combining power is 36.5 cc. per 100 cc. of plasma. Raymund[16] found the average CO_2-combining power in dogs to be 43.4 cc. per 100 cc. of plasma.

In Table II are summarized the results of a blood chemical analysis for 10 consecutive days on a normal fasting dog. There is a fall in urea nitrogen, non-protein nitrogen, and sugar. Little change is noted in the CO_2-combining power and there is no decrease in chlorides.

The blood findings with the different types of operation are grouped together. The changes following ligation of the duodenum, ligation of the duodenum and gastroenterostomy, and ligation of the ileum are shown in Tables III, IV, and V. There is a gradual rise in the chlorides and a rise in the CO_2-combining power. There are no significant changes in the non-protein nitrogenous bodies. The rise in the urea nitrogen follows closely that of the total non-protein nitrogen, and, contrary to the idea expressed by Cooke, Rodenbaugh, and Whipple, it is a good index of the rise in blood nitrogen. The bicarbonate content of the blood plasma, as shown by the CO_2-combining power, is usually doubled.

[12] Van Slyke, D. D., and Cullen, G. E., *J. Am. Med. Assn.*, 1914, lxii, 1558.
[13] Benedict, S. R., *J. Biol. Chem.*, 1922, li, 187.
[14] Gettler, A. O., *J. Am. Med. Assn.*, 1921, lxxvii, 1652.
[15] Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 473.
[16] Raymund, B., *Am. J. Physiol.*, 1920, liii, 109.

The findings in two dogs following section of the ileum at the ileocecal valve are of interest in comparison with the effects of obstructions of the upper intestinal tract (Table VI). The symptoms

TABLE I.

Blood of Normal Dogs.

Dog No.	Amount per 100 cc.							CO_2-combining power.
	Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Sugar.	Chlorides.	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*
1	35.7	7.0		2.1		116		
2	30.0	8.4		1.8		79		
3	22.8		1.3	1.3		55		31.5
4	34.9	8.9	2.0	1.9		55		38.4
5	27.8	8.9	1.4	1.2	5.7	79	380	42.1
6	23.0	8.4	1.5	1.4	5.7	79	380	43.9
7	32.3	14.0		1.5		50		32.8
8	23.6	7.9	1.1	1.1	5.0	68	420	42.8
9	27.3	10.3		1.6		109	520	38.1
10	26.5	6.1	1.0	1.6	6.3	64	410	43.0
11	23.8	6.1	1.1	1.6	4.9	63	490	43.7
12	22.2	5.6	2.8	1.8			455	34.7
13	20.5	7.5	1.6	1.4	5.8	75	510	39.3
14	40.0	9.3		1.6			460	42.1
15	26.1		2.1	1.7	7.0	82	470	43.9
16	51.8	23.4	1.6	1.5	7.1	86	470	39.3
17	51.8	26.1	2.3	1.7	7.7	52	430	42.1
18	33.3	17.8	1.5	1.5	7.0	61	470	29.1
19	40.0	23.4	1.3	1.5	6.8	54	500	27.4
20	30.0	7.0	0.6	1.4	7.0	100	460	20.8
21	22.4	7.0	0.6	1.2	7.0	61	440	33.8
22	37.5	15.9	0.6	1.4		111	450	26.3
23	28.5	9.8	1.8	1.5		83	440	32.4
24	20.8	5.1	2.0	1.6	5.7	91	490	28.7
25	25.4	10.3	1.5	1.4		73	510	43.0
Average.....	30.3	11.1	1.5	1.5	6.3	76	458	36.5

presented are similar to those seen in high obstruction. The animals live somewhat longer and show a small increase in total nitrogen. There are no significant changes in the CO_2-combining power or chlorides.

TABLE II.

Normal Fasting Dog.

Day.	Blood.								Urine.		
	Amount per 100 cc.							CO_2-combining power.	Volume.	Chlorides.	Total nitrogen.
	Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Sugar.	Chlorides.				
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	*cc.*	*gm.*	*gm.*
1	37.5	15.9	0.6	1.4		111	450	26.3	500	0.90	0.43
2	33.0	11.7	0.7	1.7	7.0	133	470	29.1			
3	25.0	9.3		1.4	5.8	87	460	34.7			
4	24.0	7.9	0.7	1.2	7.0	103	490	40.0	150	0.57	1.80
5	21.8		0.7	1.4	5.6	100	470	44.3			
6	23.0	7.0	1.5	1.7			560	39.0			
7	27.3	14.5	1.7	1.4	5.4	87	490	32.0			
8	28.0	6.1	1.7	1.3	5.6	59	490	44.7	30	0.02	0.14
9	30.6	7.0	1.2	1.3	4.9	93	500	32.4	250	0.80	2.40
11	35.3	15.8	1.5	1.3	5.5	111	520	37.2	100	0.40	1.10

TABLE III.

Dog. 24. Ligation of the Lower End of the Duodenum.

Day after operation.	Blood.								Urine.		
	Amount per 100 cc.							CO_2-combining power.	Volume.	Chlorides.	Total nitrogen.
	Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Sugar.	Chlorides.				
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	*cc.*	*gm.*	*gm.*
0	20.0	5.2	2.0	1.6	5.7	91	490	28.7	1,000	4.2	3.2
1	22.2	8.4	1.8	1.6	6.4	88	420	40.0	500	0.2	5.3
2	31.6	7.0	1.5	1.6	6.4	77	340	57.0	800	3.7	5.3
3	30.6	5.6		1.4	7.5	121	420	50.4	300	0.1	2.8
4	37.5	15.4	1.3	1.3	6.6	80	300	48.5	300	0.1	2.8
5	30.6	5.1	1.4	1.4	5.8	114	280	62.6	250	0.3	2.9
6	57.8	25.7	1.4	1.3	7.5	125	250	75.9	80	0.4	0.5
7	97.3	46.2	1.6	2.7	10.0	154	360	77.1	200	0.4	1.6
8	150.0	88.3	1.5	1.5	7.7	119	210	66.4	200	0.3	2.1

Dog 20 is of special interest. The ileum of this animal was obstructed about midway with a tape ligature. This partly cut through, leaving a partial obstruction with only a small opening. There

TABLE IV.

Ligation of the Duodenum with Gastroenterostomy.

Dog No.	Day after operation.	Blood. Amount per 100 cc. Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Sugar.	Chlorides.	CO_2-combining power.	Urine. Volume.	Chlorides.	Total nitrogen.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	*cc.*	*gm.*	*gm.*
2	0	26.3		1.3	1.3		90		24.9	170	0.7	0.8
	1	24.8	6.5	1.1	1.3		78		21.1	210	0.7	0.8
	2	61.8	31.7	2.3	2.9		66		58.0	400	2.0	1.1
3	0	25.5	6.5	1.5	3.4		104		33.4	500	1.9	2.5
	1	42.2	18.3	1.3	2.2		91		54.0	1,200	4.0	0.7
	2	67.4	27.8		3.8		78		60.7	2,000	5.4	32.0
5*	0	27.8	8.9	1.4	1.2	5.7	79	380	42.1	500	1.6	3.6
	1	29.7	9.8	1.4	1.8		94	370	58.8	500	1.6	4.3
	2	60.2	33.6	1.9	1.7		78	300	46.7	530	1.6	9.6
	3	40.0	23.6	1.4	1.5		73	300	37.4	1,200	1.7	5.5
	4	36.6	15.4	1.3	1.7		78	310	56.9	1,000	1.3	6.0
	5									700	1.3	7.7
	6	35.3	8.4	1.0	1.2		82	350	50.4	1,200	1.9	3.4
14	0	40.0	9.3		1.6			460	42.1	200	1.4	0.7
	1	27.3	12.6	2.8	1.5		159	390	38.4	800	2.7	3.2
	2	26.3	10.3					340	47.6	200	0.3	1.2
	3	36.6	18.2		2.1			290	81.0	400	0.6	0.8
	4	42.2	22.4	2.7	1.8		91	300	67.1	100	0.3	1.1
	5	107.0	74.3	3.1	2.6		175	220	47.6	200	1.2	0.8

* This dog recovered.

were no symptoms at any time. There was a marked fall in chlorides without other changes in the blood. The animal was sacrificed 3 weeks after operation. A marked hypertrophy of the ileum above the obstruction was found.

TABLE V.

Ligation of the Ileum.

Dog No.	Day after operation.	Blood. Amount per 100 cc. Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Sugar.	Chlorides.	CO_2-combining power.	Urine. Volume.	Chlorides.	Total nitrogen.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	*cc.*	*gm.*	*gm.*
8	0	23.6	7.9	1.1	1.1	5.4	68	420	42.8	10	0.2	0.1
	1	34.9	7.5		1.0	5.0	56	350	51.3	500	1.2	0.6
	2	25.4	7.5	1.0	2.0	5.4	57	370	58.9	300	0.33	3.6
	3	30.0	9.3	1.2	1.6	5.3	64	355	50.4	500	0.5	1.1
	4	40.0	17.8	2.3	1.7	4.6	87	310	51.3	500	0.7	4.6
	5	42.2	27.1	1.2	1.0		61	330	59.8			
	6	120.0	74.0	2.3	2.0		149	210	47.6	1,000	3.6	2.6
9	0	27.3	10.3		1.6		109	520	38.1	300	0.9	0.9
	1	33.0	11.2		1.9			360	42.8			
	2	30.0	12.6	1.5	1.8			270	50.4	1,000	1.0	3.3
	3	30.6	11.7	1.4	2.0	6.6	116	330	50.4	200	0.8	1.6
	4	40.0	22.4		1.6	6.1	90	290	65.5	300	0.66	5.4

TABLE VI.

Ligation of the Ileum at the Ileocecal Valve.

Dog No.	Day after operation.	Blood. Amount per 100 cc. Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Sugar.	Chlorides.	CO_2-combining power.	Urine. Volume.	Chlorides.	Total nitrogen.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	*cc.*	*gm.*	*gm.*
23	0	28.5	9.8	1.8	1.5		83	440	32.4			
	1	29.1	12.1	2.0	1.5	6.4	108	440	36.2	1,000	1.2	1.7
	2	31.2	9.3	1.4	1.4	6.2	105	420	44.7			
	3	27.7	5.1	1.4	1.2	5.4	95	440	34.2	660	0.1	0.7
	4	31.6	9.3	1.7	1.2	6.6	88	390	34.2			
	5	35.2	14.0	1.5	1.2	7.7	105	410	43.8	300	0.2	2.9
	6	40.0	15.4	1.6	1.2	7.5	125	380	49.4	100	0.1	1.7
	7	50.0	22.7	1.6	1.2	7.5	118	400	38.1	150	0.03	1.5
	8	79.0	33.6	2.0	1.2	7.5	87	390	38.1	100	0.2	1.3
	9									1,000	1.2	6.2
25	0	30.0	7.9	1.4	1.2	5.3	42	500	40.4			
	1	28.5	7.0	2.5	1.1	5.1	49	470	34.3			
	2	23.4	6.1	2.6	1.2	5.1	95	460	40.0	500	0.15	4.6
	3	21.8	5.6	3.3	1.0	6.5	49	450	43.8	500	0.10	5.7
	4	21.1	12.6	1.8	1.3	5.1	57	470	32.4	500	0.13	5.7
	5	30.6	11.2	2.0	1.2	5.1	49	500	36.2			
	6		25.2	1.6	1.1	6.1	42	500	30.5	50		

DISCUSSION.

These studies show that the first chemical changes taking place during the intoxication incident to obstruction of the upper intestinal tract are a fall in the blood chlorides and a rise in the carbon dioxide-combining power of the plasma. The rise in non-protein nitrogen does not occur until there is a marked depletion of the chlorides.

It seems evident that the fundamental change is a fall of chlorides. The rise in CO_2 is probably incidental to the chloride metabolism. There would seem to be first a loss or a fixation somewhere of the chlorine ion, leaving the sodium ion free to combine with carbon dioxide and to circulate as sodium carbonate. The level of the CO_2 in the blood plasma will, however, depend upon the rate of formation, the rate of excretion of carbonates by the kidney, and the neutralizing effect on the carbonates of acid bodies formed in the course of the intoxication. In most instances there is an actual increase in the available alkali. This is an especially important fact in connection with the treatment of such intoxications. It has been the custom with clinicians in the past to treat patients with intestinal obstruction by administration of large amounts of alkali. In certain cases tetany develops, due, it seems, to the great excess of alkali. The onset of this complication can only be hastened by giving alkali.

The changes in the chloride metabolism are the most striking feature of the intoxication due to high obstruction. The chlorides are first affected and are lost or used up in some manner. The depletion of chlorides is probably related to the increased protein destruction. The most obvious explanation for the disappearance of the chlorides is a loss by vomiting. We have cited, however, one clinical case[1] in which the blood changes were observed in the entire absence of vomiting. In several of the dogs operated upon there was practically no vomiting until just before death, although the fall in chlorides began soon after operation. The total chloride excretion in the combined urine and vomitus is low. In Dog 20 (Table VII) there was a marked fall without vomiting. To rule out vomiting completely as a cause of the fall, we studied the chlorides following intestinal obstruction in one rabbit. Rabbits cannot vomit. Before operation the non-protein nitrogen was 36.1 mg. and the chlorides 530 mg. per

100 cc. of blood. 30 hours after operation the non-protein nitrogen had risen to 108 mg. and the chlorides had fallen to 350.

It is well known that with an increase in carbon dioxide in the plasma there is a fall in the plasma chlorides due to the interchange between plasma and red cells. We made all chloride determinations, however, on whole blood, thus eliminating this interchange as a factor. It seems possible that the chlorides may be used up in the course of the

TABLE VII.

Dog 20. Partial Obstruction of the Ileum.

Day after operation.	Blood. Amount per 100 cc. Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Sugar.	Chlorides.	CO_2-combining power.	Urine. Volume.	Chlorides.	Total nitrogen.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	*cc.*	*gm.*	*gm.*
0	30.0	7.0	0.6	1.4	7.4	100	460	20.8			
1	33.3	9.8		1.4	8.1	49	490	48.6	800	2.6	2.6
2	33.0	14.9	1.1	1.3	8.9	111	430	38.4	1,500	1.8	9.9
3	26.5	10.7	0.6	1.7	7.3	70	480	45.8	250	0.6	1.5
4	22.7	6.5	0.6	1.5	7.7	76	310	50.4	35	0.1	0.8
5	22.5	8.4		1.6	7.1		370	58.9	50	0.02	1.0
6	30.0					78	430	57.6	200	0.04	8.0
7	24.4	7.9		1.5			340	36.2	100	0.03	1.0
8	28.2	10.7	1.3	1.5	6.6	80	380	46.6	30	0.01	0.7
9	30.6	14.0	1.8	1.4	6.2	56	380	51.3	60	0.2	0.2
10	34.1	13.5	1.6	1.4	6.9	84	390	41.9	200	0.04	2.2
11									600	0.18	4.8
12	32.2	13.1	1.4	1.2	5.5	108	420	41.9	600	0.18	4.8

increased tissue destruction. Against this is the fall in chlorides before the nitrogen destruction becomes evident. It is true also that there may be an attempt to protect the body protein by a change in reaction since autolysis is not so active in alkaline media, and it is possible that the toxic agent may act through autolysis. And finally, the possibility that the chlorides may play a direct protective part must be considered. Further studies are under way to determine the exact part played by the chlorides and will be reported in a later paper.

If it is essentially protective the use of chlorides in treatment may be highly important. The results of such treatment both in man and in experimental animals, as contrasted with the results after administration of glucose, water, and diuretics, will be reported later.

The relation between the non-protein nitrogen and the urea nitrogen is of interest. The results indicate clearly that when the determinations are made by the methods outlined, the urea nitrogen is a reliable index of the level of non-protein nitrogen in the blood.

The amino-acid nitrogen shows little change. This with a normal urea metabolism, we believe, negatives entirely the idea recently expressed by Werelius[17] that hepatic insufficiency is the immediate cause of death in obstruction.

The sugar shows no significant change. The uric acid and creatinine show nothing unusual.

These studies emphasize the importance of blood chemistry in the study of intestinal intoxications. Heretofore, results of experiments have been determined largely by symptoms and by whether or not death ensued. The symptoms are due to changes which are reflected in the blood long before the symptoms arise. This is well shown in Dog 5 (Table IV) and Dog 20 (Table VII). In these animals there were no symptoms at any time. One showed well marked chemical changes in the chlorides and non-protein nitrogen. The other showed changes in the blood chlorides due to a partial obstruction as demonstrated by autopsy.

It is of special interest to note the differences in salt metabolism when the obstruction is high up in the intestinal tract and when it is lower down. With the first mentioned condition there is a depletion of the chlorides with a resulting alkalosis, whereas with the latter there is no fall in chlorides and no alkalosis.

The chloride metabolism of the upper intestinal tract intoxications is similar to that found by Peabody[18] in acute lobar pneumonia due to the pneumococcus, and in serum disease by Rackemann, Longcope, and Peters.[19]

[17] Werelius, A., *J. Am. Med. Assn.*, 1922, lxxix, 535.

[18] Peabody, F. W., *J. Exp. Med.*, 1913, xvii, 71.

[19] Rackemann, F. M., Longcope, W. T., and Peters, J. P., *Arch. Int. Med.*, 1916, xviii, 496.

SUMMARY AND CONCLUSIONS.

A study of the non-protein nitrogen, urea nitrogen, uric acid, creatinine, amino-acid nitrogen, sugar, and chlorides of the blood and the CO_2-combining power of the plasma in normal dogs, and in dogs after different types of intestinal obstruction, is reported.

Following ligation of the duodenum, ligation of the duodenum with gastroenterostomy, and ligation of the upper half of the ileum, a fall in chlorides and a rise in the non-protein nitrogen and urea nitrogen of the blood and in the CO_2-combining power of the plasma occur. The uric acid, creatinine, amino-acid nitrogen, and sugar show no significant changes. The fundamental change is a fall in chlorides followed by an alkalosis. The degree of alkalosis depends upon the rate of formation of carbonate, rate of excretion by the kidneys, and extent of neutralization of the carbonate by acid bodies formed during the intoxication.

The fall in chlorides is probably due to a utilization of the chlorine ion in the course of the intoxication. It is suggested that this use of chlorine is a protective measure on the part of the body.

There are indications that high intestinal obstruction should not be treated by the administration of alkalies.

The urea nitrogen is a good index of the protein destruction.

Ligation of the ileum at the ileocecal valve is followed by little increase in nitrogen and no change in the chlorides or CO_2-combining power of the plasma.

The close similarity of the blood findings in intestinal obstruction, acute lobar pneumonia, and serum disease suggests that these widely different conditions may have a common chemical basis.

CHEMICAL CHANGES IN THE BLOOD OF THE DOG AFTER PYLORIC OBSTRUCTION.

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In a chemical study of the blood after high intestinal obstruction in dogs it has been shown that the characteristic changes are a fall in chlorides, an increase in the non-protein nitrogen, and usually an increase in the carbon dioxide-combining power of the plasma.[1]

These findings have certain points in common with the chemical changes reported by several observers in the study of the so called gastric tetany occurring after pyloric obstruction. MacCallum, Lintz, Vermilye, Leggett, and Boas[2] have shown that following section of the pylorus in dogs a fall in chlorides and an increase in the CO_2-combining power of the plasma occur. They conclude that the fall in chlorides is due to the loss of hydrochloric acid in the gastric juice through vomiting and that the symptoms are the result of the alkalosis. They noted that the symptoms could be relieved by injections of sodium chloride. McCann[3] found that following operations on the stomach which exclude acid from the duodenum, tetany develops which is similar to parathyroid tetany. In both conditions he found an increase in the CO_2-combining power of the plasma. He concludes that tetany is a condition of alkalosis. Hastings, Murray, and Murray[4] have also made certain studies on the blood after ligating the pylorus in an attempt to produce gastric tetany. All of their dogs developed an alkalosis. They found also that the calcium content of the blood was slightly higher than normal, that the chlorine and sodium were decreased, and that the sulfur and phosphorus were increased. In making a study of clinical tetany Grant[5] reports two cases with obstruction at the pylorus in which there was an increase in the CO_2-combining power of the plasma. In one case he found a marked reduction in chlorides in the blood.

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[1] Haden, R. L., and Orr, T. G., *J. Exp. Med.*, 1923, xxxvii, 365.

[2] MacCallum, W. G., Lintz, J., Vermilye, H. N., Leggett, T. H., and Boas, E., *Bull. Johns Hopkins Hosp.*, 1920, xxxi, 1.

[3] McCann, W. S., *J. Biol. Chem.*, 1918, xxxv, 553.

[4] Hastings, A. B., Murray, C. D., and Murray, H. A., Jr., *J. Biol. Chem.*, 1921, xlvi, 223.

[5] Grant, S. B., *Arch. Int. Med.*, 1922, xxx, 355.

The investigations quoted have been made with the hope of throwing light on the origin of tetany. Since it has been shown that a calcium deficiency is probably the cause of the tetany occurring after parathyroidectomy, work has chiefly been done on the inorganic salt metabolism. No attention has been paid to the protein metabolism or its possible relation to the inorganic salts. Our findings in intestinal obstruction as regards the chlorides and CO_2-combining power are essentially the same as those reported in tetany following pyloric obstruction. We have shown, however, that in intestinal obstruction there is an intimate relation between the chlorides and the protein metabolism and that both in turn are dependent upon the action of the toxic agents. This has led us to make complete studies of the blood following pyloric obstruction since it seemed probable that there was a protein destruction which might have an important bearing on the tetany.

Method.

All experiments have been made on dogs. All operations were done under ether anesthesia with aseptic technique. Obstruction of the pylorus was effected by ligating with a piece of narrow tape. The dogs were kept in metabolism cages. No food was given after operation and usually none for 48 hours preceding operation. Water was given *ad libitum*. Vomitus was collected with the urine for the estimation of the chlorides. Blood was obtained for analysis from the jugular vein just before operation and at 24 hour intervals thereafter.

The non-protein nitrogen, sugar, and creatinine of the blood were determined by the method of Folin and Wu,[6] the amino-acid nitrogen by the method of Folin,[7] uric acid by the method of Benedict,[8] urea nitrogen by the Van Slyke and Cullen[9] modification of the Marshall method, CO_2-combining power by the method of Van Slyke,[10] and the

[6] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.
[7] Folin, O., *J. Biol. Chem.*, 1922, li, 377.
[8] Benedict, S. R., *J. Biol. Chem.*, 1922, li, 187.
[9] Van Slyke, D. D., and Cullen, G. E., *J. Am. Med. Assn.*, 1914, lxii, 1558.
[10] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.

chlorides on the tungstic acid filtrate after the technique suggested by Gettler.[11] The chlorides in the urine were determined by a modified Volhard-Arnold method.

OBSERVATIONS.

The dogs vomited freely after operation. Typical tetanic convulsions were observed in only one animal (No. 16), although muscle tremors were present in all. The animal developing tetany was taken directly from the stock cage and lived only 72 hours after operation. It showed a very high blood non-protein nitrogen before death.

The other animals lived from 4 to 9 days. At autopsy there was no peritonitis. The stomach was not markedly dilated. The ligature was always in place. It was found, however, that the obstruction was not complete. An opening through the pylorus large enough to admit a lead pencil was regularly present. The dog living only 4 days showed multiple abscesses in the spleen, kidneys, and retroperitoneal lymph glands. The blood findings, which are not so characteristic as in the other animals, may have been influenced by the infection. The results of the chemical studies are summarized in Table I.

In each animal a marked fall in the chlorides of the whole blood, a rise in CO_2-combining power of the plasma, and a marked rise in total non-protein nitrogen and urea nitrogen occur. The uric acid, creatinine, sugar, and amino-acids show practically no change. The total chloride excretion in the urine and vomitus was never large.

DISCUSSION.

It is quite evident that following pyloric obstruction there is a marked protein destruction in addition to the alkalosis and fall in chlorides. The fall in chlorides precedes the rise in blood nitrogen and there is seemingly a close relation between the two. The increase in blood nitrogen and in the nitrogen destruction appears before there are symptoms of tetany. It is apparent that the possibility must be considered that the tetany is due to the protein split-products instead of to the alkalosis. The only dog in the series which showed tetany

[11] Gettler, A. O., *J. Am. Med. Assn.*, 1921, lxxvii, 1652.

TABLE I.

Chemical Findings in Pyloric Obstruction.

Dog No.	Day after operation.	Blood. Amount per 100 cc. Total non-protein nitrogen.	Urea nitrogen.	Uric acid.	Creatinine.	Amino-acid nitrogen.	Sugar.	Chlorides.	CO_2-combining power.	Urine and vomitus. Volume.	Chlorides.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*vol. per cent*	*cc.*	*gm.*
12	0	22.2	5.6	2.8	1.8			455	34.7	1,300	0.33
	1	24.6			1.6		115	450	27.2	400	0.40
	2	30.0	7.9		1.6	6.6	137	550	52.3	150	0.21
	3	36.0	10.3	2.6	1.5			465	54.1	2,500	2.00
	4	38.9	14.0	2.6	1.5	8.1	189	300	65.3	1,500	2.10
	5	57.8	33.6		1.9		200	340	58.8	500	1.35
	6	77.0	43.3	2.5	1.9	7.1	135	340	71.8	1,300	0.78
	7	70.6	43.3	2.2	1.8	7.2	175	220	85.7	1,200	1.78
	8	75.0	35.0	2.1	1.7	7.0	172	220	76.4		
	9	81.8	54.6	2.1	1.6	6.4	109	270	83.8	1,300	1.56
13	0	20.5	7.5	1.6	1.4	5.8	75	510	39.3	300	0.60
	1	22.8	8.4	2.1	1.5	5.0	81	470	51.4	800	2.40
	2	28.2	7.9	2.2	1.5	5.6	103	420	57.9	1,250	3.60
	3	33.2	16.4	2.0	1.5	7.3	87	290	69.0	2,000	3.20
	4	112.8	58.8		1.9	7.9	82	280	80.1	800	1.12
	5	180.0	106.2	2.3	2.4	7.2	102	230	86.0	1,500	2.50
	6	266.0	162.5		2.7			110	101.7	400	0.48
	7									700	0.35
15	0	26.1		2.1	1.7	7.0	82	470	43.9	500	1.75
	1	27.7	12.6	2.2	1.6	7.1	109	420	54.1	3,500	14.00
	2	38.9	18.6	0.7		6.6		340	55.1		
	3	60.0	35.0	2.3	1.9	6.6	79	260	80.1	1,500	5.85
	4	40.6	29.4	2.7	0.9	7.2	192	240	100.7	500	0.50
16	0	51.8	23.8	1.6	1.5	7.1	86	470	39.3		
	1	23.6	12.6	2.3	1.6	6.2	109	400	54.1	1,500	4.65
	2	98.4	49.0	2.1	1.5	6.3	115	290	66.2	200	0.28
	3	173.4	119.7	2.5	3.0	6.2	161	150	83.8	200	0.28

had the lowest CO_2-combining power, but a marked and rapid protein destruction. In only one dog (No. 15) is there a large chloride excretion in the combined urine and vomitus. It seems that a loss of

chloride in the gastric juice as an explanation for the low blood chloride and the alkalosis can consequently be ruled out. The possibilities involved in the relation between the fall in chlorides and the increase in nitrogen have been discussed in the preceding paper.[1]

The nitrogen destruction explains also the increase in sulfur and phosphorus found by Hastings, Murray, and Murray, since both substances come from protein. We believe that these studies show that the intoxication following pyloric obstruction is essentially the same as that following high intestinal obstruction. It is evident that a study of the tetany following pyloric obstruction must include the protein as well as the inorganic salts. The possibility must be considered that the condition is primarily a disturbance in the protein and only secondarily in the inorganic salts.

CONCLUSIONS.

Chemical studies of the blood and urine of four dogs following pyloric obstruction are reported.

The observations of other workers that a fall in chlorides and a rise in CO_2-combining power of the plasma occur, are confirmed.

There is also a marked rise in the non-protein nitrogen of the blood, consisting mainly of urea nitrogen and undetermined nitrogen.

The fall in chlorides is not due to the loss of chlorides in the gastric juice. The chlorine is probably bound somewhere in the process of protein destruction.

There is a close relation between the fall in chlorides and the protein destruction.

A study of tetany should include the protein metabolism as well as that of the inorganic salts, since it seems possible that the tetany is due to protein split-products and not to the alkalosis.

The chemical changes following pyloric obstruction are essentially the same as those following high intestinal obstruction.

IMMUNITY STUDIES OF ROCKY MOUNTAIN SPOTTED FEVER.

I. Usefulness of Immune Serum in Suppressing an Impending Infection.

By HIDEYO NOGUCHI, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, November 6, 1922.)

Rocky Mountain spotted fever has been for the past 20 years the subject of study by various workers, including members of the United States Public Health Service. The discoveries of Wilson and Chowning,[1] McCalla,[2] King,[3] Ricketts,[4] Maver,[5] Moore,[6] Cooley,[7]

[1] Wilson, L. B., and Chowning, W. M., The so-called "spotted fever" of the Rocky Mountains, *J. Am. Med. Assn.*, 1902, xxxix, 131; Studies in *Piroplasmosis hominis*. "Spotted fever" or "tick fever" of the Rocky Mountains, *J. Infect. Dis.*, 1904, i, 31.

[2] McCalla, L. P., Direct transmission from man to man of the Rocky Mountain spotted (tick) fever, *Med. Sentinel*, 1908, xvi, 87.

[3] King, W. W., Experimental transmission of Rocky Mountain spotted fever by means of the tick. Preliminary note, *Pub. Health Rep., U. S. P. H.*, 1906, xxi, 863.

[4] Ricketts, H. T., The transmission of Rocky Mountain spotted fever by the bite of the wood-tick (*Dermacentor occidentalis*), *J. Am. Med. Assn.*, 1906, xlvii, 358; Further observations on Rocky Mountain fever and *Dermacentor occidentalis*, 1067; The rôle of the wood-tick (*Dermacentor occidentalis*) in Rocky Mountain spotted fever, and the susceptibility of local animals to this disease—a preliminary report, 1907, xlix, 24; Further experiments with the wood-tick in relation to Rocky Mountain spotted fever, 1278; Observations on the virus and means of transmission of Rocky Mountain spotted fever, *J. Infect. Dis.*, 1907, iv, 141.

[5] Maver, M. B., Transmission of spotted fever by other than Montana and Idaho ticks, *J. Infect. Dis.*, 1911, viii, 322; Transmission of spotted fever by the tick in nature, 327.

[6] Moore, J. J., Time relationships of the wood-tick in the transmission of Rocky Mountain spotted fever, *J. Infect. Dis.*, 1911, viii, 339.

[7] Cooley, R. A., Preliminary report on the wood tick. *Dermacentor sp.* Sixth Annual Report of the State Entomologist of Montana, *Montana Agric. Coll. Exp. Station, Bull. 75*, 1909, 95; Tick control in relation to the Rocky Mountain spotted fever, *Montana Agric. Coll. Exp. Station, Bull. 85*, 1911.

Parker,[8] and Wolbach,[9] have proved that the disease is transmitted to man by a certain wood tick, *Dermacentor venustus*, which receives the virus from infected wild rodents, principally gophers or Columbian ground squirrels and jack-rabbits (Wilson and Chowning; Parker). Ricketts[10] was the first to transmit the disease from man to guinea pigs and monkeys by injecting the blood drawn from spotted fever patients and, after having fed normal ticks on the infected guinea pigs, to reproduce the disease again in normal guinea pigs by allowing the infected ticks to feed on the animals. The virus of Rocky Mountain spotted fever can be maintained in the laboratory for an indefinite time by animal passage, which makes its experimental study possible. Several strains of the passage virus[11] maintained in the guinea pig were employed in the present series of studies on immunity.

Ricketts and Gomez[12] found that the serum from guinea pigs which had recovered from the disease would protect normal guinea pigs from infection when administered within 2 to 3 days after the injection of the infecting material. The strength of the serum was such that 0.3 to 0.5 cc. was required to protect against 1 cc. of virus (1,000 infecting doses), and these investigators were unable to enhance the

[8] Parker, R. R., Tick control program for 1919, *Montana State Bd. Entomol., Circular 1*, 1919; The control of Rocky Mountain spotted fever in the Bitter Root Valley, *Montana State Bd. Entomol., Circular 1* (*Revised*), 1921. *3rd Biennial Rep., Montana State Bd. Entomol., 1917–18*, 1919; *4th Biennial Rep., Montana State Bd. Entomol., 1919–20*, 1921.

[9] Wolbach, S. B., Studies on Rocky Mountain spotted fever, *J. Med. Research*, 1919–20, xli, 1.

[10] Ricketts, H. T., The study of "Rocky Mountain spotted fever" (tick fever?) by means of animal inoculations. A preliminary communication, *J. Am. Med. Assn.*, 1906, xlvii, 33.

[11] I am indebted to Dr. L. D. Fricks, of the United States Public Health Service, Dr. S. B. Wolbach, of Harvard Medical School, and Dr. R. R. Parker, of the Montana State Board of Health and United States Public Health Service, for their cooperation in supplying me with virus and ticks on numerous occasions since 1916.

[12] Ricketts, H. T., and Gomez, L., Studies on immunity in Rocky Mountain spotted fever. The preventive and curative action of a serum for spotted fever, and the inefficiency of sodium cacodylate as a curative agent for this disease in guinea-pigs, *J. Infect. Dis.*, 1908, v, 221.

potency by repeatedly injecting an immune animal with virus. They accordingly drew the tentative conclusion that the usefulness of the serum in human cases would be practically nil, especially since the amount required would be prohibitive when translated into terms of human body weight. Ricketts' work was not completed, however, and there seem to be no experimental data to indicate how a serum of this kind might be developed so as to be of practical value. The purpose of the experiments recorded here was to study this problem and if possible to produce an immune serum of at least sufficient potency to be useful when the time of infection is definitely known and the injection of serum can be made soon after the inoculation of the virus, as when a laboratory worker is bitten by an infected tick or accidentally inoculates himself with an instrument charged with infected material.

Although the serum of guinea pigs which have passed through a severe infection has a degree of neutralizing power when injected at the same time as the virus, it is not strong enough to check the course of the infection when introduced 24 to 48 hours later. In the present series of experiments, however, it has been found that the neutralizing power of the serum is considerably increased by one or two more subsequent inoculations of the virus, in citrated blood, into the convalescent guinea pig, and sera prepared in this way are capable of modifying the course of the disease into a non-fatal one or, in some instances, of suppressing the infection completely, provided the serum is administered during the incubation period.

The small size of the guinea pig precludes the possibility of utilizing its immune serum for human cases, but the principle developed from the experiments with the serum of guinea pigs suggested that larger animals might be capable of furnishing an equally potent immune serum in correspondingly greater amounts.

Ricketts and Gomez,[12] and later Heinemann and Moore,[13] reported having infected horses by injecting large quantities of virus from guinea pigs or monkeys. Although the horses showed some fever on the 3rd or 4th day after inoculation, it never reached 104°F. and usually lasted only 1 day. The serum from the horses, however, was stated to possess some neutralizing power as compared with normal horse serum.

[13] Heinemann, P. G., and Moore, J. J., Experimental therapy of Rocky Mountain spotted fever, *J. Infect. Dis.*, 1912, x, 294.

EXPERIMENTAL.

The writer's experiments with horses have so far been unsuccessful. Two adult horses were given intravenous injections of blood from infected guinea pigs in doses ranging from 20 to 50 cc. at intervals of 3 to 5 days, and a colt 6 months old received similar injections of larger amounts (35 to 95 cc.) of the virus. The rise of temperature to 103–104°F. which occurred on the 3rd or 4th day after inoculation was interpreted as being due to the introduction of a large quantity of blood corpuscles from a foreign species. The animals never showed symptoms suggestive of spotted fever, and the serum on test was found to have only slight neutralizing power for the virus; 1 cc. was necessary to neutralize about 10 M.L.D. when administered simultaneously.

In view of the facts that adult ticks feed on horses and cattle, and that a certain number of ticks collected at random prove to be infective, it was thought of interest to test the serum of horses which had been pastured in infected districts. Serum from a horse which had been pastured for several years in the Bitter Root Valley was obtained[14] and tested against the virus. As shown in Table I this serum (No. 1) in a dose of 3 cc. gave complete protection against 1 cc. (approximately 100 M.L.D.) of the spotted fever virus when injected simultaneously. Of the other three sera subsequently obtained from horses pastured in the same region, one (No. 2), which was contaminated on arrival, failed to give any protection in a dose of 3 cc., while Sera 3 and 4 had definite neutralizing power, the animals receiving 3 cc. of the serum simultaneously with 100 M.L.D. of the virus recovering after a typical febrile reaction without scrotal or skin lesions.

TABLE I.

Administration of Serum from Horses Pastured for Several Years in the Bitter Root Valley of Montana to Guinea Pigs Inoculated with 100 M. L. D. *of the Rocky Mountain Spotted Fever Virus.*

Serum No.	Date.	Amount.	Result.	Remarks.
	1922	*cc.*		
1	Feb. 3	3	No infection.	Tested for immunity after 40 days and found to be completely immune.
1	Apr. 2	3	" "	Died of intercurrent infection before test for immunity could be carried out.
2 (contaminated on arrival).	" 25	3	Typical infection.	Died in 13 days.
3	" 25	3	" fever for 4 days.	Recovered without lesions.
4	" 25	3	Typical fever for 5 days.	" " "

[14] The serum was obtained through the kindness of Dr. W. J. Butler and Dr. Hadleigh Marsh, of the Montana Livestock Sanitary Board.

The only animal which yields a useful serum, both in potency and quantity, is the rabbit. In the first place, the rabbit is susceptible to the virus of spotted fever. The infection is usually non-fatal, the febrile period lasting for 6 to 9 days, though it is sometimes shorter or longer. The scrotal skin may show the typical capillary thrombosis and subsequent necrosis. After recovery the animal becomes completely resistant to subsequent infection, and 0.5 to 0.1 cc. of the serum, collected 2 weeks after disappearance of symptoms, is capable of rendering non-fatal an infection produced by 100 M.L.D. of the virus, the test animal in every instance being the guinea pig. By another intravenous injection of virulent blood, *e.g.* of 4 cc., or 400 M.L.D., the serum can be raised in potency five- to tenfold, and in some instances to such a titer that 0.1 cc. will neutralize 1,000 M.L.D. The reinforcing injection is given 2 weeks after complete recovery of the animal, and the immune serum is drawn 9 days later. A serum, whether convalescent or reinforced, is sufficiently strong for practical use when it is capable in a dose of 0.1 cc. of neutralizing 100 M.L.D. of the virus. As will be seen from the results recorded in Tables II to IV, the immune rabbit serum prevents the development of the infection or renders it non-fatal when administered within the period of incubation—the earlier the administration of the serum the more complete the suppression of the infection.

Male guinea pigs of 500 gm. body weight were used. The virus[15] consisted of the citrated blood from the heart of guinea pigs on the 4th or 5th day of fever and was inoculated subcutaneously. 1 cc., equivalent to 100 M.L.D., was given in Experiment 1, 0.1 cc. (10 M.L.D.) in Experiment 2, and 0.01 cc. (1 M.L.D.) in Experiment 3. Eighteen guinea pigs were used in each series, two animals being inoculated with the immune serum immediately after the injection of the virus, and two at the end of 24, 48, 72, and 96 hours, and 5, 6, and 7 days. The remaining two animals served as controls without serum. The immune serum used was a mixture of sera from several immunized rabbits and was given in a dose of 1 cc. in Experiments 1 and 2 and 0.1 cc. in Experiment 3. The titer of the pooled serum was such that when given simultaneously 1 cc. completely neutralized 100 M.L.D.,

[15] These experiments were carried out with a strain furnished by Dr. Parker.

TABLE II.

Experiment 1. Administration of Immune Rabbit Serum to Guinea Pigs Inoculated with 100 M. L. D. *of the Rocky Mountain Spotted Fever Virus.*

Amount of serum, 1 cc. uniformly.

Guinea pig No.	Interval before serum administration.	Symptoms before serum administration.	Symptoms after serum administration.
1A	Immediately after inoculation.	None.	None.
1B	Immediately after inoculation.	"	"
	hrs.		
2A	24	"	"
2B	24	"	"
3A	48	"	Temperature rose to 104.5° F. in 7 days; remained high for 24 hrs. Recovered.
3B	48	"	High fever developed in 9 days; lasted for 4 days. No scrotal lesions. Recovered.
4A	72	"	High fever on 6th day; remained for 4 days. No scrotal lesions. Recovered.
4B	72	"	High fever for 3 days after 5th day. No scrotal lesions. Recovered.
5A	96	"	High fever continued for 5 days. No scrotal lesions. Recovered.
5B	96	Temperature 105°F. in morning.	High fever in 7 days, lasting 48 hrs. No scrotal lesions. Recovered.
	days		
6A	5	Fever for 2 days.	No abatement of temperature. Typical course. Died in 16 days.
6B	5	High fever for a day.	Fever continued at 105–106.5°F. for 8 days. Died in 17 days.
7A	6	" " (105–107° F.) for 2 days.	No fall in temperature. Typical course. Died in 13 days.
7B	6	High fever for 2 days.	No reduction in fever. Typical course. Died in 13 days.

TABLE II—*Concluded.*

Guinea pig No.	Interval before serum administration.	Symptoms before serum administration.	Symptoms after serum administration.
	days		
8A	7	High fever for 3 days.	Apparently no effect on the course of infection. Died in 13 days.
8B	7	" " " 3 "	No beneficial effect. Typical course. Died in 12 days.
Controls without serum.			
9A	Temperature 105°F. after 96 hrs. and remained high for 5 days; fell to subnormal on the 11th day. Scrotum, ears, and feet showed typical lesions. Died in 12 days.		
9B	Rise of temperature to 104.5°F. in 96 hrs.; continued high for 7 days. Died in 13 days.		

0.1 and 0.01 cc. modified the infection to a non-fatal one, while 0.001 cc. exerted no influence upon the course of the infection. The serum was given subcutaneously.

As Table II shows, 1 cc. of the serum, given within 24 hours from the time of introduction of a massive amount (100 M.L.D.) of the virus into the body of the guinea pig, is capable of suppressing the infection; when given later, but before 96 hours (near the time of onset of the disease), this quantity does not suppress the infection but renders it non-fatal. After 5 days, that is when the disease is already manifest, no benefit seemed to be derived from administration of the serum, the animals dying within 12 to 17 days (the controls died within 12 to 13 days).

The results in the second series (Table III) are similar, except that, owing probably to the smaller quantity of the virus used (10 M.L.D.), the infection was completely suppressed in all animals which received the serum within the first 48 hours. All those which received it after 48 hours, but within 96 hours, survived, after passing through a mild infection without any scrotal or other cutaneous lesions. Administration of the serum later than 5 days invariably failed to prevent a fatal outcome, the animals dying just as the controls did.

In the third series (Table IV), in which only 1 M.L.D. was used to infect the animals, the protective effect of the immune serum was

TABLE III.

Experiment 2. Administration of Immune Rabbit Serum to Guinea Pigs Inoculated with 10 M.L.D. *of the Rocky Mountain Spotted Fever Virus.*

Amount of serum, 1 cc. uniformly.

Guinea pig No.	Interval before serum administration.	Symptoms before serum administration.	Symptoms after serum administration.
10A	Immediately after inoculation.	None.	None.
10B	Immediately after inoculation.	"	"
	hrs.		
11A	24	"	"
11B	24	"	"
12A	48	"	"
12B	48	"	"
13A	72	"	High fever after 5 days; lasted only 3 days. Recovered.
13B	72	"	High fever after 5 days; lasted 4 days. Recovered.
14A	96	"	High fever developed in 7 days; lasted 2 days. No scrotal lesions. Recovered.
14B	96	"	High fever in 7 days; lasted 5 days. No scrotal lesions. Recovered.
	days		
15A	5	First sign of fever (105° F.).	Fever continued for 7 days. Typical infection. Died in 12 days.
15B	5	Beginning of fever.	High fever continued for 8 days. Died in 13 days.
16A	6	" " high fever.	Fever of typical course. Died in 13 days.
16B	6	High fever for 24 hrs.	Fever remained high for 7 days. Died in 14 days.
17A	7	" " " 48 "	No influence on the course of the infection. Died in 13 days.
17B	7	" " " 48 "	Fever for 5 days. Typical course. Died in 12 days.
Controls without serum.			
18A	Temperature 105.2°F. in 5 days; remained high for 7 days. Died in 13 days.		
18B	Temperature 105°F. in 5 days; remained high for 6 days. Died in 12 days.		

TABLE IV.

Experiment 3. Administration of Immune Rabbit Serum to Guinea Pigs Inoculated with 1 M.L.D. *of the Rocky Mountain Spotted Fever Virus.*

Amount of serum, 0.1 cc. uniformly.

Guinea pig No.	Interval before serum administration.	Symptoms before serum administration.	Symptoms after serum administration.
19A	Immediately after inoculation.	None.	None.
19B	Immediately after inoculation.	"	"
	hrs.		
20A	24	"	"
20B	24	"	"
21A	48	"	"
21B	48	"	"
22A	72	"	"
22B	72	"	"
23A	96	"	"
23B	96	"	Fever in 7 days; lasted 5 days. Recovered.
	days		
24A	5	"	Fever in 7 days; lasted 48 hrs. Recovered.
24B	5	Temperature 105° F. in the morning.	Fever lasted for 8 days. Typical scrotal lesions. Died in 16 days.
25A	6	Temperature 106° F. in the morning.	Fever remained high for 7 days. Typical infection. Died in 14 days.
25B	6	None.	Temperature 106°F. in 9 days; lasted 48 hrs. Recovered.
26A	7	"	Fever in 9 days; remained high 3 days. Recovered.
26B	7	Temperature 105.5° F. for 24 hrs.	Fever and scrotal lesions typical. Died in 17 days.
Controls without serum.			
27A	Temperature rose to 105.5°F. in 5 days; lasted for 9 days. Scrotal skin necrotic; ears hardened. Died in 18 days.		
27B	Temperature 105°F. in 7 days; lasted for 8 days. Scrotal lesions typical. Died in 16 days.		

much more pronounced than in the first two series. Here the infection was completely suppressed when 0.1 cc. of the serum was given within 72 to 96 hours. In one of each set of two animals treated

with the serum after 5, 6, and 7 days respectively, the infection was mild, undoubtedly because the serum was given before the animals showed any fever, while they were still in the incubation period, which is decidedly longer in the case of 1 M.L.D. than when 10 or 100 M.L.D. are inoculated.

DISCUSSION AND SUMMARY.

From the results of the experiments presented it is evident that in guinea pigs an early administration of immune rabbit serum will suppress the infection; that is, if it is given within the period of incubation, the effect being proportionately greater the earlier the serum is administered. Almost no beneficial effect is observed when the serum is given after the onset of the disease. In the animals inoculated with 10 to 100 M.L.D. the incubation period is shorter than when 1 M.L.D. is injected; nevertheless 1 cc. of the immune serum saved the animals as late as 96 hours from the time of the introduction of the virus into the system. When administered within 24 hours in the case of 100 M.L.D. and within 48 hours in the case of 10 M.L.D., the serum completely neutralized the virus, and the animals escaped infection altogether. On the other hand, the same quantity of the serum only modified the infection into a non-fatal one when given a day or two later. In the animals which were inoculated with 1 M.L.D. the incubation period was a day or two longer, and the neutralizing effect of the serum was much more powerful. Here animals were saved as late as 5, 6, and 7 days and with a much smaller quantity of the serum (0.1 cc.).

As to the usefulness of such an immune serum in human cases, the relative susceptibility of man and the guinea pig must first be considered. In a large number of experimental infections carried out with guinea pigs in the past 6 years almost never has a naturally refractory animal been encountered. The mortality is nearly 80 per cent with most strains, although as low as 50 per cent with some. The strain used in the present study caused death in nearly 80 per cent of the animals. Hence the susceptibility of guinea pigs is at least as great as that of man, in whom the mortality in the Bitter Root Valley is estimated to be about 70 per cent.[1,9]

The relative length of the incubation period in guinea pig and in man is another point which requires analysis. In guinea pigs it varies somewhat according to the number of passages, being as short as 3 days when 100 M.L.D. or more of an adapted virus are inoculated. On the other hand, when the infection is the result of 1 M.L.D. or the bite of an infected tick, the incubation period is much longer, being 5, 6, or 7 days in the former and 7 to $8\frac{1}{2}$ days in the latter instance, as with the present strain. In man the infection is brought on by the bite of an infected tick, and the period of incubation varies from 3 to 10 days but is usually 7 days; *i.e.*, it is about the same as in guinea pigs infected with 1 M.L.D. Hence we may regard the susceptibility of man and the guinea pig as nearly equal.

The final point to be considered is the quantity of the immune serum that may be recommended for use in human cases. To prevent the infection in a guinea pig weighing 500 gm., 0.1 cc. of the serum was sufficient. This quantity protected the animal against 1 M.L.D. even as late as 5, 6, or 7 days. Calculated on this basis, 16 cc. of the serum would be required for a man weighing 80 kilos (about 160 pounds); that is, 16 cc. of an immune rabbit serum, administered before onset of the disease, should theoretically be sufficient to save a man of average weight against an infection brought about by the bite of an infected tick or by a laboratory accident. It would probably be best to administer the serum intravenously. The titer of the immune serum should be previously determined in guinea pigs, and 1 cc. should neutralize 100 M.L.D. completely and 0.1 and 0.01 cc. render the infection non-fatal. Such a serum is easily produced in rabbits (a rabbit weighing 2,500 gm. will yield 50 to 60 cc. of the serum) and probably will remain active a year or longer when kept at refrigerator temperature.

CONCLUSIONS.

A potent immune serum against the virus of Rocky Mountain spotted fever can be produced in the rabbit.

By early administration of such an immune serum the virus introduced into the guinea pig can be prevented from multiplying and causing fatal infection. When given within the period of incubation the suppression of the infection is assured, but after the onset of the disease the serum has no beneficial effect.

In view of the comparative susceptibility of man and the guinea pig it is recommended that in every instance when the bite of a tick gives reason to suspect a possible infection with spotted fever, or when a person accidentally inoculates himself with the virus, about 0.2 cc. of the serum per kilo of body weight (or 0.1 cc. per pound) be injected immediately, preferably intravenously. For the average adult about 16 cc. should be given.

STUDIES ON THE TOTAL BILE.

I. The Effects of Operation, Exercise, Hot Weather, Relief of Obstruction, Intercurrent Disease, and Other Normal and Pathological Influences.

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(Received for publication, September 29, 1922.)

By a method already described[1] the entire bile output of dogs can be obtained over long periods of time, unexposed to gall bladder activity, and uncontaminated with wound secretion or the product of inflamed ducts. Aided thereby we have studied anew several of the classical, unsolved problems of bile secretion. The first step, of necessity, has been to familiarize ourselves with the day to day variations in the bile due to intercurrent causes. To these, which are often striking and significant, the present paper will be devoted.

Method.

The dogs were kept throughout in roomy metabolism cages, and were given no yard exercise because of its possible influence upon the bile, a precaution justified by the findings, as will be shown. Long experience had convinced us that normal dogs kept and fed as described remain in good health. Following the operative insertion of the collecting apparatus, the bile was removed from the collecting balloon every morning at about the same hour. Cultures were made of it at once in bouillon and on agar plates. Its appearance and consistency were then noted, the quantity measured, and a 1 cc. specimen was mixed with Hooper and Whipple's[2] acid alcohol (49, 99, 199, or 399 cc., according to the bilirubin content of the specimen) and set aside for a colorimetric reading. The remainder was stored in the ice box. By duplicate mixtures with the acid alcohol, made on successive days, each specimen was quantitated at least once with those immediately preceding and following it, and against the same portion of inorganic standard.[3] Thus the individual readings became closely linked.

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[1] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 11.

[2] Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916, xl, 332.

[3] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 47.

Just before the bile was removed from the balloon each day the hemoglobin percentage of the blood was ascertained by the Newcomer method with a specimen from a marginal ear vein; and immediately after the bile evacuation, food for the 24 hour period was given the animals. The routine bilirubin and hemoglobin determinations were made by different workers, each unaware of the other's findings, to rule out the subjective element.

Ordinarily the diet consisted of a mixture of cooked lean meat and bread in regular proportion; but, for special purposes, this was often varied with a ration of cooked lean meat and liver, or bread, milk, and bones. The amount eaten in the 24 hours, as of water drunk, was measured.

Each day the urine was tested for bilirubin (Gmelin reaction, and, for quantitative purposes, Hooper and Whipple's[2] modification of the Salkowski test). For the detection of urobilin and urobilinogen, the spectroscopic method of Wilbur and Addis[4] was employed, and for bile salts Hay's test. The stools were frequently searched for stercobilin (Schlesinger's reaction), to detect a possible reopening of the original duct channel; but it may be said at once that this complication was never encountered.

The Effects of Operation.

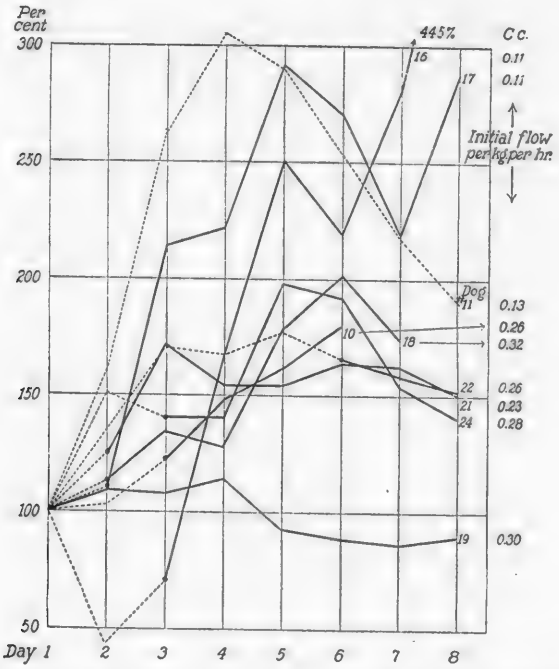
The literature contains no quantitative studies on the bile secreted in the period immediately after the operation whereby a biliary fistula is produced in animals.

During the first few days after the operative insertion, under ether, of our collecting apparatus and especially during the first 24 hours, the bile was nearly always much more scanty and more heavily pigmented than that secreted later on (Text-figs. 1 and 2). It was also more syrupy. The bile of the second 24 hours after operation was usually thicker than that of the first, sometimes even ropy, despite an almost regular, and frequently considerable, increase in amount; and on one occasion at least, a temporary obstruction at this time was referable to such cause (Text-fig. 3). Since recovery from the operation was rapid, and the animal during the second 24 hours was ordinarily in excellent condition, the peculiar consistency of the bile may be referred rather to a local reaction on the part of the traumatized ducts than to general influences.

In Text-fig. 1 the postoperative changes in the bile amount are expressed in terms of the output of the first 24 hours. The actual initial rate of bile flow per kilo per hour is given at the right margin of

[4] Wilbur, R. L., and Addis, T., *Arch. Int. Med.*, 1914, xiii, 235.

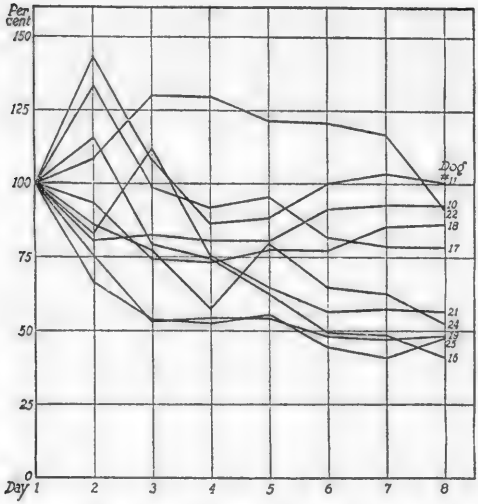
the chart. It will be observed that, in general, when this initial flow was least the relative increase later was greatest, as would inevitably follow from the fact that the later, or "normal," rate of bile flow is subject to much less variation than is the initial rate of flow.



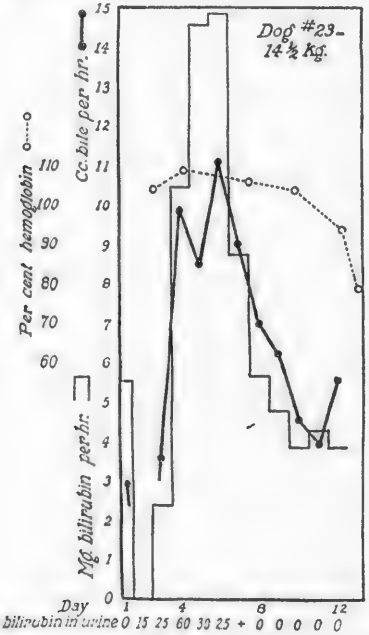
TEXT-FIG. 1. Postoperative changes in rate of bile flow as expressed in percentages of the 1st day's yield. The dotted ruling indicates that the animal was fasting.

For a day or so after the operation the dogs as a rule took little food. Since 1852 it has been known that fasting animals yield less bile than do those full fed.[5] Here was a possible cause for the small postoperative output. To determine whether it was the actual one, some of the animals were denied food for several days after operation,

[5] Bidder, F., and Schmidt, C., Die Verdauungssäfte und der Stoffwechsel. Eine physiologisch-chemische Untersuchung, Mitau and Leipsic, 1852.



TEXT-FIG. 2. Postoperative changes in bilirubin output as expressed in percentages of the 1st day's yield.



TEXT-FIG. 3. Postoperative obstruction due to viscid bile.

though given free access to water. The bile, at first scanty, underwent an increase later as in the well fed animals (Text-fig. 1). A lessened ingestion of food after operation will not, then, account for the findings. Nor probably will a water lack, for the animals always drank freely after operation, and the hemoglobin per cent of the blood did not change.

The scanty first bile was, as already stated, much more pigmented than the later secretion. The fact is well attested that with the increased pigmentation of the bile that follows upon a sudden liberation of hemoglobin in the body the quantity of the secretion may become temporarily less.[6] In certain of our dogs the operative trauma caused some extravasation of blood into the tissues, and from this hemoglobin must have been set free. In other instances, though, in which special attention was given to such a possibility and hemostasis carefully maintained, it can be practically ruled out; and nevertheless the bile was scanty.

Altogether the results incline us to the belief that the small output of bile immediately after operation is attributable directly to the influence of this latter upon the liver,—though the nature of the influence remains to be determined. Many authors have noted that in laparotomized dogs kept under ether for the collection of bile, none may be forthcoming;[7] and Hooper and Whipple have made the interesting observation that upon the introduction of a rubber drainage tube into a cholecystostomy opening bile secretion sometimes ceases for many minutes.[2]

One potential cause for the high pigment content of the bile in the first days after operation as compared with that secreted later has been mentioned above, namely a liberation of hemoglobin from extravasated blood. That this was occasionally at least an actual cause was demonstrated by the course of the bilirubin output in instances in which blood was known to have been lost into the tissues. A marked temporary rise in the output of pigment was then to be noted between the 2nd and 5th postoperative days, that is to say

[6] Stadelmann, E., Der Icterus und seine verschiedenen Formen. Nebst Beiträgen zur Physiologie und Pathologie der Gallensecretion, Stuttgart, 1891.

[7] For example, Wertheimer, E., *Arch. physiol. norm. et path.*, 1892, iv, series 5, 577; Neilson, N. M., and Meyer, K. F., *J. Infect. Dis.*, 1921, xxviii, 510.

during precisely the period when the extravasated blood was breaking down. A similar rise was observed later on, in two dogs, during the absorption of small hematomas resulting from jugular puncture.

The findings thus far described were obtained in animals yielding a sterile bile and rapidly recovering from the operative procedure. The results were far different in certain others in which the liver had been damaged with mercuric chloride carried into the peritoneal cavity upon the operator's gloves. The collecting balloon had been left within the abdomen in these instances, but its position may be dismissed from account in the present connection since in our experience bile is ordinarily secreted freely into bags so left. The mercury poisoning resulted in death of the animals after about 2 days; and there was outspoken parenchymatous and fatty degeneration of the liver at autopsy. In these cases almost no bile was secreted from the time of operation; in one of them none whatever during the 48 hours that elapsed before death. At the postmortem examination, in this case, the larger bile channels were found undilated, but in the lumen of each lay a thick cord of dark brown, semisolid, mucinous material. That the hepatic cells continued to secrete bile pigment was shown by a marked jaundice of the liver.

In several early experiments in which the collecting balloon had been left within the abdomen, infection of the biliary tract occurred; and in one of these in which a mixture of organisms was present, bile ceased to be secreted into the bag, and its place was taken by a clear, glairy, tenacious material like white of egg, obviously a product of the inflamed ducts. In this instance only a part of the liver, and this part atrophying as result of long standing local diversion of the portal flow,[8] was drained by the bag; and the pressure under which the bile was secreted was low, as always after such a change in the circulation.[9] But the finding suffices to illustrate one method whereby catarrhal icterus may be supposed to develop.

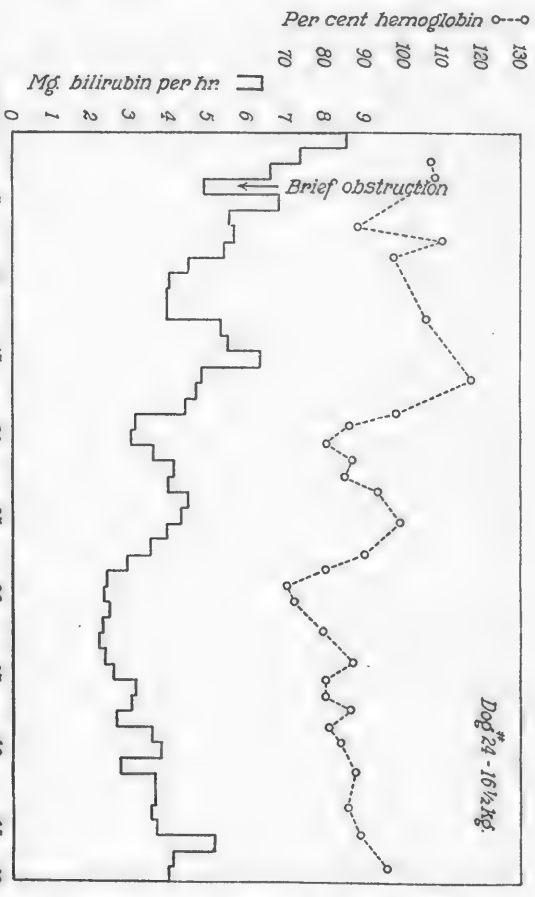
The Later Output.

Within 3 to 7 days as a rule the bile has increased to approximately the amount yielded during the weeks that follow if the conditions

[8] Rous, P., and Larimore, L. D., *J. Exp. Med.*, 1920, xxxi, 609.

[9] Rous, P., and Larimore, L. D., *J. Exp. Med.*, 1920, xxxii, 249.

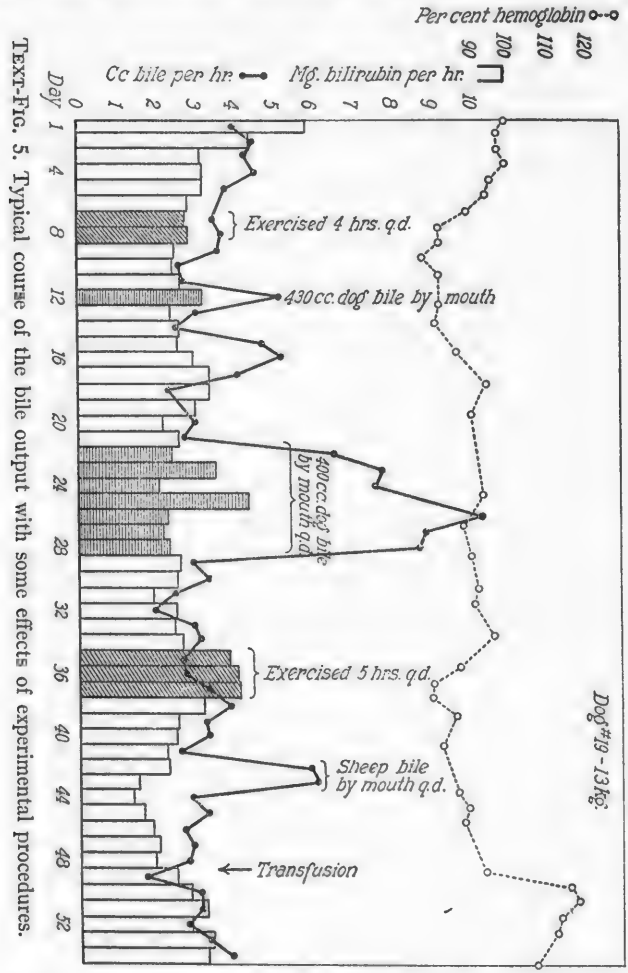
are not varied; but often as many as 8 or 10 days elapse before the pigment output falls to the "normal" of the succeeding period. The reasons for this will be discussed in a later paper; suffice it here that the gradual absorption of the hemoglobin from extravasated blood


Per cent hemoglobin
70 80 90 100 110 120 130
Mg. bilirubin per hr.
0 1 2 3 4 5 6 7 8 9
Day 1 5 10 15 20 25 30 35 40 45 48
Brief obstruction
Dog 24 - 16½ kg.

TEXT-FIG. 4. Relation between the hemoglobin percentage of the blood and the bilirubin output.

is not the sole one. Always, despite an apparent lively health, a well healed wound, and a varied diet, there develops, more or less gradually, a mild but obdurate anemia of secondary type. The drop in the hemoglobin percentage incident thereto is accompanied by one

in the bilirubin output. That animals markedly anemic yield sometimes a bile notably poor in pigment is known.[10] But it is surprising to find that intercurrent variations in the hemoglobin percentage


Per cent hemoglobin
90
100
110
120
Cc bile per hr.
Mg. bilirubin per hr.
0 1 2 3 4 5 6 7 8 9 10
Day 1 4 8 12 16 20 24 28 32 36 40 44 48 52
Exercised 4 hrs. q.d.
430 cc. dog bile by mouth
400 cc. dog bile by mouth q.d.
Exercised 5 hrs. q.d.
Sheep bile by mouth q.d.
Transfusion
Dog 49-1913.

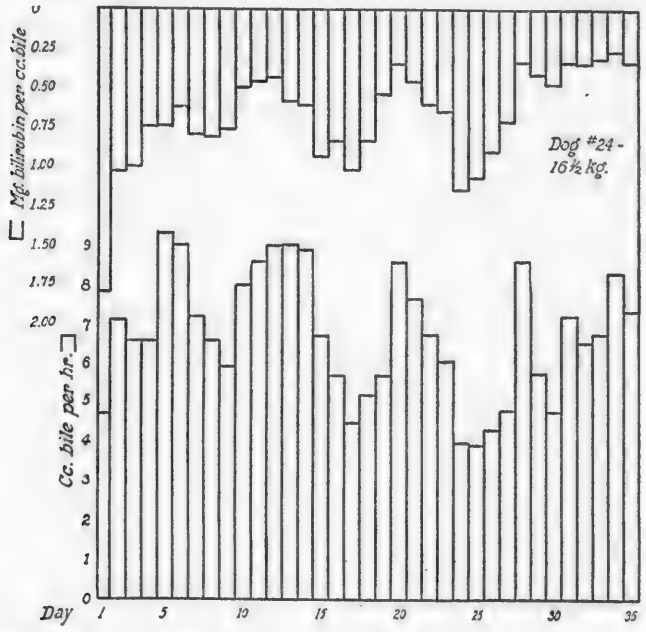
TEXT-FIG. 5. Typical course of the bile output with some effects of experimental procedures.

are closely paralleled by similar variations in the bilirubin yield. In many instances slow wave-like changes in both are to be noted (Text-fig. 4). Their importance in the interpretation to be accorded

[10] Whipple, G. H., and Hooper, C. W., *Am. J. Physiol.*, 1917, xliii, 258.

to data on the bilirubin output is obvious. In the absence of complicating influences, this output changes remarkably little from day to day, a fact which cannot be too much stressed. A typical curve, with incidental variations referable to experiment, is given in Text-fig. 5.

The bile quantity, by contrast to that of the pigment, undergoes sudden and great variations under normal circumstances. It follows,



TEXT-FIG. 6. Reciprocal relation between the fluid output of bile and its pigment concentration.

if a uniform output of pigment is to be maintained, that when the bile is copious it will be weak in pigment per cubic centimeter, and, when scanty, strong. This is strikingly true (Text-fig. 6). A like reciprocal relationship seems to exist normally between the quantity of the secretion and its mucinous constituent, judging from gross changes in the consistency of the fluid. A scanty bile is usually syrupy or even ropy, and a profuse one watery. The average specimen of hepatic duct bile, as we have observed it, is only tacky not syrupy.

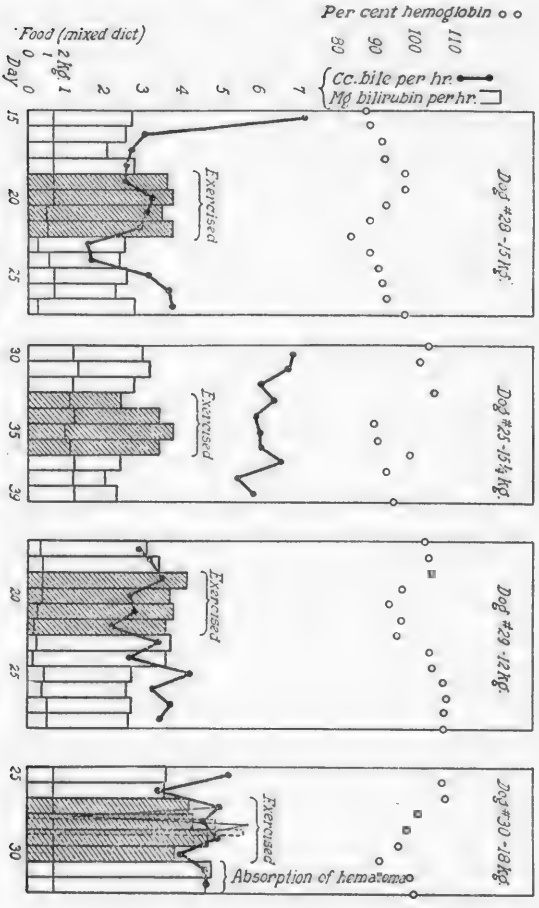
The Pigment Quantity.

Prior to the development of the mild anemia just mentioned, the bilirubin output corresponds closely with that recorded by Stadelmann.[6] This observer, studying three animals, found that the quantity of pigment in 24 hours varied from 4.2 to 10.2 mg. per kilo of dog and averaged between 6 and 7 mg. In nine dogs we have noted a range of from 5.2 to 11 mg. and an average of 7.4 mg. at such times as the hemoglobin percentage approximated the normal. These are to be considered as maximal figures for the individuals in question, since during much of the period of bile collection there was anemia and associated with it a low pigment output. Hooper and Whipple report an average output of about 9 mg. per kilo (1 mg. per pound of animal in 6 hours); but their animals had outdoor exercise each day, and, as will now be shown, may with reason be presumed to have made and broken down more blood.

The Effects of Exercise.

Broun[11] has shown that vigorous exercise of dogs previously kept under sedentary conditions leads to an increased blood destruction, as further that the daily turnover of blood in active animals must be greater than in quiet ones. Since bile pigment is supposed to come from destroyed blood, vigorous bodily activity may well influence the daily output. It was for this reason that our animals were kept caged. A number of experiments on the effects of running upon treadmills were carried out with them; and the results demonstrate plainly that such unprepared for exercise during 4 or 5 hours of each day causes, at the same time with the drop in the hemoglobin percentage already noted by Broun, a marked absolute increase in the bilirubin output (Text-figs. 5 and 7). Further data on the point will be recorded in a later paper. There can be no doubt of its general importance in relation to bilirubin figures. Curiously enough the quantity of the bile is not essentially altered by exercise. In the case of Dog 30 (Text-fig. 7) the output for the 6 hours in which exercise went on was separately examined on two occasions.

[11] Broun, G. O., *J. Exp. Med.*, 1922, xxxvi, 481; 1923, xxxvii, 113, 187, 207.



TEXT-FIG. 7. Effect of exercise on the bilirubin output.

The Bile Quantity.

That the bile quantity may vary greatly from day to day and even more from hour to hour is known to every student of the secretion.

According to Heidenhain's[12] summary, dogs secrete from 2.9 to 36.1 cc. per kilo in 24 hours. He considered 23 to 35 cc. as normal. Stadelmann[6] found a

[12] Heidenhain, R., in Hermann, L., Handbuch der Physiologie, Leipsic, 1883, v, pt. 1, 367.

range of from 8.8 to 21 cc., figures obtained by 12 to 24 hour collections from animals on a mixed diet. The hourly variations are frequently enormous. Bidder and Schmidt[5] demonstrated that fasting animals yield far less bile than those well fed, and Nasse[13] showed that the output is considerably greater on a meat diet than on one of carbohydrates (bread, potatoes). A host of later investigators has reaffirmed these facts. Voit,[14] Dastre,[15] and others have constructed curves indicating the dependence of the secretory rate on the time of feeding.

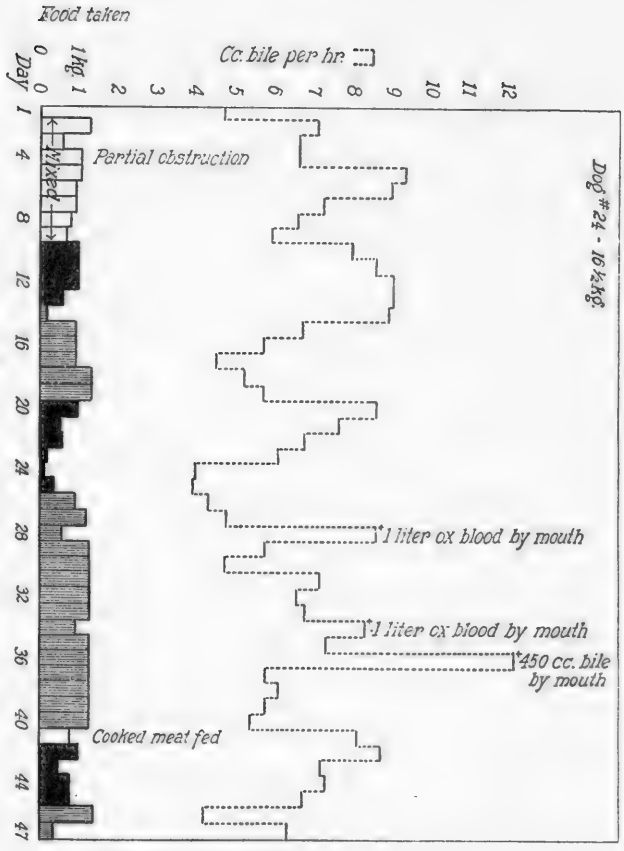
In our animals, lively, well nourished, and subsisting upon a varied diet, the average yield of bile was greatly less than that observed by any of the investigators just named. In seven uncomplicated instances the individual minimum ranged from 1 to 7 cc. per kilo in 24 hours, and the maximum from 8 to 14 cc. The minimum was registered on warm days when the animal ate little, or partook mainly of starchy food, and the maximum when meat, with sometimes a little cooked liver, had been given. The striking influence of the character of the food was over and over again manifest (Text-fig. 8). The average output on a mixed diet of bread and meat ranged from 3.5 to 9.5 cc. Some individuals consistently secreted less bile than others. For example, one vigorous dog of 13 kilos not infrequently put out as little as 3.3 cc. per kilo in 24 hours, never more than 9.6 cc., and averaged about 5 cc.; whereas another of 12 kilos yielded from 7 to 13 cc. with an average of 10 cc., or twice as much. In both cases the liver and duct system proved normal at autopsy.

During intercurrent illness unassociated with jaundice, the bile output may become extremely small. In No. 23, an animal of $11\frac{1}{2}$ kilos, yielding 6 to 7 cc. per hour when in health, only 0.23 cc. per hour, and this dark and ropy, was secreted during 27 hours of a severe coryza which was recovered from. It is difficult to be certain of the precise cause for such lessenings. Usually the dog has for some days eaten but little. They are, it is interesting to find, influenced but little by the administration through the stomach tube of a mixed dog bile that under better circumstances acts in the same animal as a marked cholagogue.

[13] Nasse, H., Commentatio de bilis quotidie a cane secreta copia et indole, Marburg, 1851, cited by Voit.[14]

[14] Voit, C., *Z. Biol.*, 1894, xxx, 523.

[15] Dastre, A., *Arch. physiol. norm. et path.*, 1890, ii, series 5, 800.

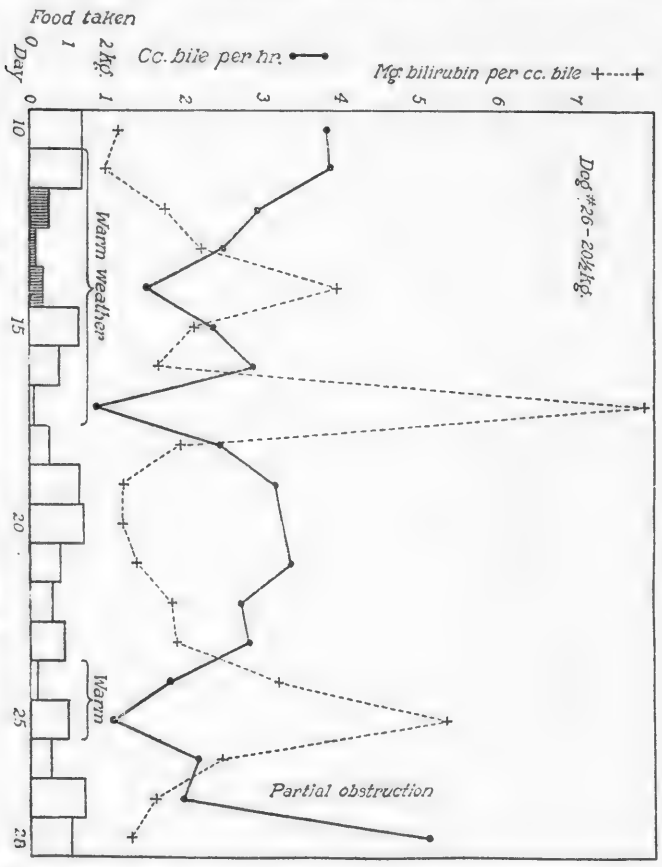


TEXT-FIG. 8. Influence on the bile amount of the character and quantity of the food.

The Effects of Hot Weather.

When the weather is hot the secretion of bile may strikingly diminish, even if the food intake remains considerable as is not usually the case. It was under such circumstances that our minimal "normal" figures were obtained. For example (Text-fig. 9), in a shaggy terrier of 20½ kilos the rate of secretion fell with the onset of warm weather from the already low quantity of 4 cc. per kilo in 24 hours to slightly less than 1 cc. The animal obviously suffered from the heat, panting

much and moving little; yet when its coat had been clipped, another period of warm weather, better borne, caused again a falling off of the bile to 1 cc. per kilo. The animal always had free access to water.



TEXT-FIG. 9. Change in bile amount caused by warm weather.

The Pigment Concentration.

During hot weather, when the bile output is notably small, the amount of pigment in it per cc. may be as much as 8 mg., whereas the average "normal" is considerably less than 1 mg., sometimes as little as 0.3 mg. Only through the elaboration of such different specimens is the bilirubin output maintained at the usual level.

After operations entailing some blood loss into the tissues, the scanty, first bile may contain as much as 4 mg. of pigment per cc. The total output later, when the extravasate is breaking down, may rise to 19 mg. per kilo in 24 hours, nearly three times the average "normal;" but, since the bile is then relatively copious, the pigment concentration per cubic centimeter is not great. Under other pathological conditions it is sometimes enormous. In a dog dying of pneumonia after 17 days of bile collection the black, ropy, last specimen held 19.7 mg. of bilirubin in each cc. Jaundice had developed. In Dog 23, during the coryza without jaundice, already referred to which caused the bile quantity to fall to about one-twenty-fifth of its "normal," the pigment concentration rose temporarily to 9 mg. per cc.

The Output after the Relief of Obstruction.

Most previous workers have noted far greater variations in the daily pigment output of healthy dogs than have we, a difference without doubt attributable to the methods they employed. Consecutive 24 hour collections over long periods of time have heretofore been undertaken only exceptionally, and then with the animal in a sling, that is to say under conditions which soon change it, as all admit, into an abnormal creature.[16] Collections for 6 hours or less out of each 24 have been the rule. And the fistula of Schwann has regularly been employed, an arrangement which favors, and is frequently followed by, recurring temporary closure of the cholecystostomy opening in the intervals between bile collections, with intermittent obstruction as result.[17,18] Under such circumstances large variations in the bilirubin output are to be expected.

We have studied the characters of the bile secreted after the relief of obstruction. The method employed for bile collection in the present work permits of total obstruction during several days merely by clamping the drainage tube for that period.

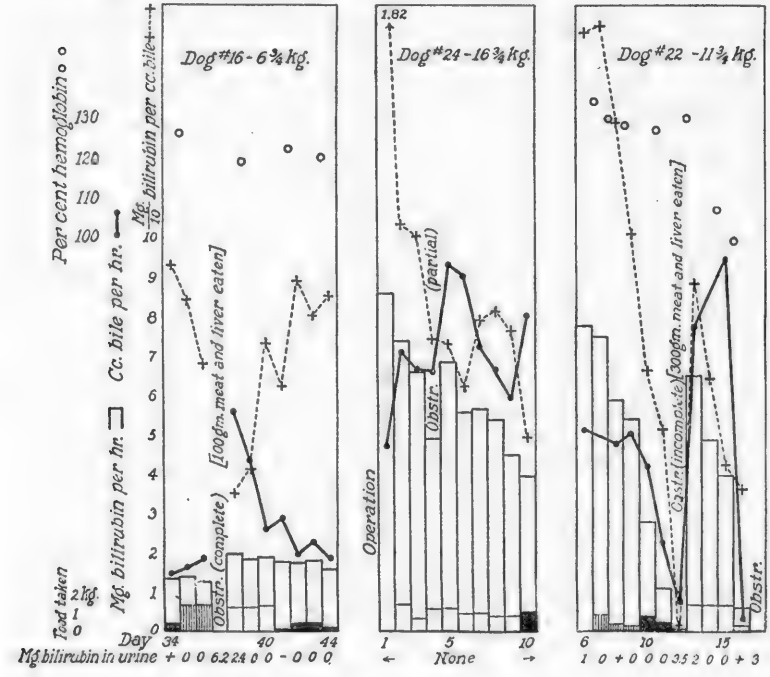
[16] The observations of Dastre by a balloon method constitute a special instance. Their results have no bearing on the subject now under discussion.

[17] von Kölliker, A., and Müller, H., *Verhandl. physik.-med. Ges. Würzburg*, 1855–56, vi, 435.

[18] Wisner, F. P., and Whipple, G. H., *Am. J. Physiol.*, 1922, lx, 119.

There exist in the literature no experiments upon the theme, though clinical records dealing with it are numerous, since nearly every surgeon of experience has had to produce a biliary fistula to relieve obstruction of the common duct. Such records usually state that a profuse flow of bile followed relief of the obstruction, with sometimes the further note that the bile was thin, and occasionally that a colorless fluid flowed forth at first, the so called "white bile" that is really a product of the ducts.[19]

Our first observations were made inadvertently.



TEXT-FIG. 10. The bile after relief from obstruction.

Dog 16,—a female fox-terrier of 6¾ kilos,—was given treadmill exercise for the first time on the 37th day of bile collection. During it the dressings slipped, bending the drainage tube sharply upon itself, with result that there followed 24 hours of total obstruction. Some characters of the subsequent bile output are recorded in Text-fig. 10.

Dog 24,—a mongrel, male hound of 16¾ kilos,—was accidentally subjected to partial obstruction through twisting of the drainage tube on the 4th day after operation, that is to say at a time when the bile pigment output was rapidly falling from the high postoperative level (Text-fig. 10).

[19] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 75.

Dog 22,—a white bull-terrier, male, of 11¾ kilos,—secreted bile in the usual amounts for some days after operation, and then the quantity rapidly diminished. On the assumption that angulation of the drainage tube within the abdomen was responsible several centimeters of the tubing was pulled forth. There followed an extremely copious bile flow during several days after which the obstruction reasserted itself, this time permanently (Text-fig. 10).

In each of these instances the bile flow was abundant upon relief of the obstruction, and there was a considerable transient increase in the output of bilirubin; but the fluid increase so far preponderated in two of the cases that the pigment concentration per cubic centimeter actually fell below the previous level. In the third case the conditions do not admit of a decision upon the point.

Systematic observations were now begun upon the consequences of obstruction, but unfortunately at a time when warm days were frequent, with, as result, notable intercurrent diminutions in the bile flow. Furthermore, extraneous circumstances made it necessary to kill several of the animals while in excellent condition, before the observations upon them had been completed. The data, nevertheless, are sufficient, and the findings clear-cut (Text-figs. 11 and 12).

Obstruction was maintained for only 24 hours. It seemed inadvisable to use longer periods because of deposition of bilirubin in the tissues, malaise, blood changes, and other inevitable complications. In the dog the kidney threshold for hemoglobin is so low,—in contrast to the human threshold,—that bilirubin regularly appears in the urine before enough has accumulated in the blood to tint the plasma or give the diazo reaction.[20] Bile salts appear in the urine later than pigment. Bilirubinuria, then, is the most delicate indicator of the retention of bile within the body of the dog. It often appears in slight degree in normal dogs after a 24 hour fast. Fortunately our animals ate well.

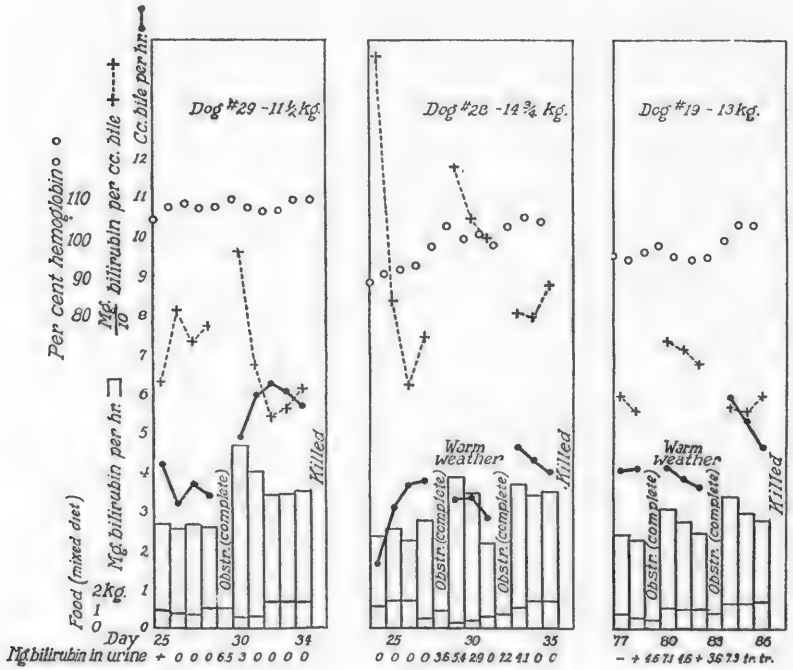
The abruptly produced biliary obstruction appeared to cause our animals no discomfort whatever. During its continuance they usually retained appetite and were lively and cheerful. The development of jaundice after ligation of the common duct of dogs is ordinarily slow, bile pigment not occurring in the urine until the 2nd day, and occasionally even later.[21] But in the intubated animals of the present work, in which no operation was required to induce obstruction, bilirubi-

[20] McMaster, P. D., and Rous, P., *J. Exp. Med*, 1921, xxxiii, 731.

[21] Whipple, G. H., and Hooper, C. W., *Am. J. Physiol.*, 1916, xl, 349.

nuria became pronounced within the first 24 hours, demonstrating that some portion of the pent up secretion had already been resorbed from the liver.

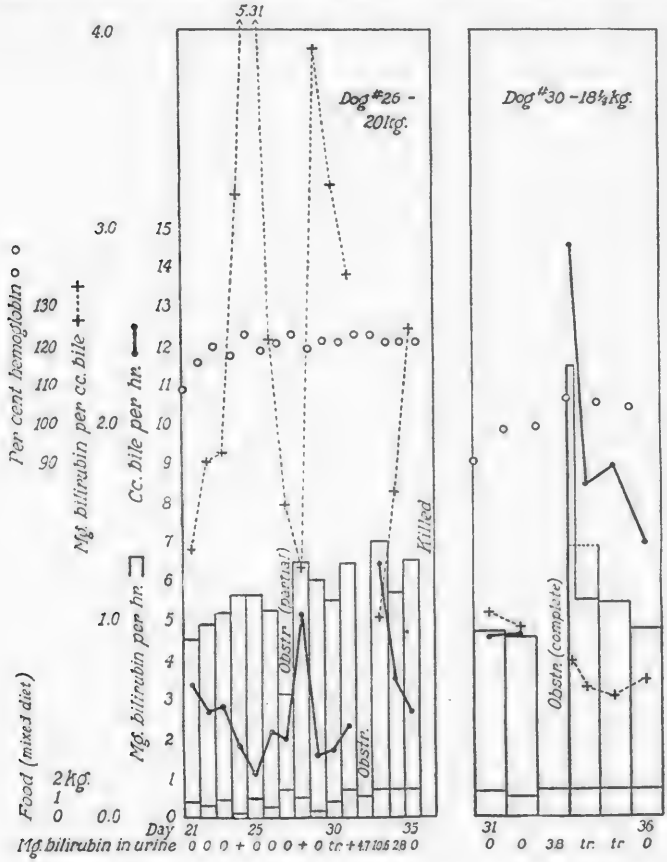
The charts show changes in the bile similar to those already remarked upon. The relief of obstruction was followed by a copious flow, save when the weather was warm; and the thin fluid had a low


Per cent hemoglobin
Mg. bilirubin per cc. bile
Cc. bile per hr.
Mg. bilirubin per hr.
Food (mixed diet)
Day
Mg. bilirubin in urine
Dog #29 - 11½ kg.
Dog #28 - 14 ¾ kg.
Dog #19 - 13 kg.
Obstr. (complete)
Killed
Warm weather
+ 0 0 0 6 5 3 0 0 0 0
0 0 0 0 3 6 5 4 2 9 0 12 4 1 0 0
- + 4 6 7 1 4 5 + 3 6 7 3 tr. tr.

TEXT-FIG. 11. The bile after relief from obstruction.

pigment concentration despite an increased total content of it. The fluid output often remained above the previous level for 48 hours, but tended, like that of the pigment, to fall rapidly to the previous level. It should be said that throughout the observations and a considerable preliminary period a standard ration of cooked meat and bread was fed, in order to rule out a possible influence of diet upon the

pigment quantity.[21] In one instance (Dog 30) the bile for the first 6 hours following the resumption of flow was collected separately. The amount secreted during this brief time was extremely large, and the



TEXT-FIG. 12. The bile after relief from obstruction.

bilirubin quantity also, but the pigment concentration per cubic centimeter was less than before the experiment.

A special interest pertains to the quantity of pigment put forth in the bile during the period of readjustment, since there are indications that bilirubin formation may be to some extent inhibited during

obstruction.[22] The interpretation of our data bearing on the point is complicated by the loss of a small amount of pigment in the urine, and furthermore by intercurrent variations in the hemoglobin percentage of the blood, which, as already stated, find a reflection in variations in the bilirubin output. Several of the experiments were performed at a time when the animals were slowly recovering from a mild anemia consequent on treadmill exercise performed while they were out of training. But allowing for these sources of error it would seem, as the charts indicate, that as much extra pigment was put out after relief of the obstruction as if its manufacture had been unimpaired throughout.

In two cases, obstruction was incomplete, entailing in the one instance (Dog 26, Text-fig. 12, first obstruction) the retention of an amount of pigment such as would ordinarily have been manufactured in about 12 hours, and in the other (Dog 24, Text-fig. 10) the yield of perhaps 8 hours. In neither case did bilirubinuria develop, and the existence of the obstruction might have gone unperceived had it been accidental in character. The point is not without importance in relation to the data obtained by bile collection from fistulas of the ordinary sort which are subject to intermittent closure.[23]

DISCUSSION.

In the present paper, as in those designed to follow it, attention has been centered on the amount of the bile and its pigment content. The bile salts are known to be derived, in part at least, from the food; and dog bile, unlike human, contains but little cholesterol, with several possible sources for it, among them the desquamated cells of the biliary tract and the food. Bilirubin has been generally supposed to have but one source, namely the hemoglobin of destroyed red cells, although recently Whipple and his collaborators have brought forward evidence which they believe shows that some of it is derived from ingested carbohydrates.[21] However this may be,—and the point is taken

[22] Whipple, G. H., Hooper, C. W., and Robscheit, F. S., *Am. J. Physiol.*, 1920, liii, 203.

[23] Pelkan, K. F., and Whipple, G. H., *J. Biol. Chem.*, 1922, l, 513.

up in a paper published herewith,[24]—the problem of the relationship between normal blood destruction and the bilirubin output remains an important one. The present communication may be looked upon as a preamble to work on the theme.

The quantity of bile secreted daily by our animals was on the average far less than that recorded by other observers, and in individual instances enormously less, despite the exceptionally good condition of the animal. This is attributable to several causes.

The observers referred to employed the open fistula method of bile collection, of which a heavily infected biliary tract[22] and cholangitis are the usual results.[23] And it is probable that sometimes a considerable part of the secretion which they collected as bile was actually the product of inflamed ducts. An instance in point is to be found in one of the dogs upon which Hooper and Whipple[2] made extensive observations (Dog 16–6). A correlation of the scattered protocols of the various experiments performed with this animal shows that between the 14th and 25th days after the operation whereby a fistula was established, bile secretion amounted to from 42 to 69 cc. in a standard 6 hour period, and averaged 58 cc. Throughout the later observations the amount steadily increased, without alterations in food or surroundings to account for the change, until, when 9 weeks had passed, the yield varied between 95 and 112 cc. with an average of 103 cc. for three successive collections. 2 days thereafter the dog died with convulsions. Pelkan and Whipple state that chronic cholangitis was usual in their animals.[23]

Many authors mention the frequency with which recurring biliary obstruction complicates bile collection by the open fistula method. Hooper and Whipple[2] refer in their autopsy protocols to a dilated condition of the ducts,—further evidence of obstruction. In the present paper it has been shown that 12 hours yield of bile may be retained without bilirubinuria, in other words without clinical evidence of obstruction, and furthermore that relief from obstruction is followed by a notably profuse flow of bile. Certain of the high figures on bile output previously reported can be referred to this cause. Yet another cause is to be found in the custom to let dogs lick away from the fistula orifice such bile as is secreted in the long periods between collections. For bile by mouth is a notable cholagogue. In this connection the fact should be stressed that none of the records

[24] Rous, P., Broun, G. O., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 421.

on bile flow, neither ours nor the previous ones, can be considered as truly representative of the rate of normal secretion. For the diversion of the bile from the intestinal tract does away with an effective stimulus to further secretion.

If the biliary tract of dogs with an open fistula remained normal one would expect the bile obtained therefrom to be somewhat less in amount, not greater, than is yielded by the method we have employed, for the gall bladder forms a link in the tract of the open fistula, and this organ normally acts to concentrate the bile.[3] But under the circumstances of the fistula the gall bladder suffers with the rest of the duct system, and loses its concentrating faculty, as is clearly shown by the accumulation of "white bile" within the passages when the fistulous opening closes for a day or so.[17] The fluid accumulation in an hydropic gall bladder is admittedly the product of the diseased walls of the viscus. The same sort of fluid must frequently mingle with the bile from an open fistula and go to swell its total to a greater or less extent.

The ordinary variations in the rate of bile flow from day to day are great, and under exceptional circumstances, as we have shown, may be extreme. One of our dogs, weighing $11\frac{1}{2}$ kilos, secreted only 6 cc. during 27 hours of coryza, but a few days later, after recovery, put forth, during a period of bile feedings, 422 and 390 cc. on successive days, that is to say from 65 to 70 times as much. In this instance there were, for reasons which need not here be gone into, unusual fluctuations in the output of pigment, but alterations in the bile quantity almost as extreme as those mentioned often occur without change in the 24 hour pigment yield. As has already been pointed out, this is kept approximately constant by means of enormous variations in the pigment concentration of the bile per cubic centimeter. The more one follows the changes in quantity of the secretion in relation to the pigment content, the more one gains the impression that the variations in the fluid bulk of the bile are extraneous to, superimposed upon, the essential secretory activities of the liver in relation to the bile solids. No matter what the bile quantity may be, great or small, increased by a protein diet or bile feeding, or lessened by warm weather, fasting, or disease, approximately the same daily quota of bilirubin is put forth in it. And Rovsing's belief

that, during some diseases unassociated with biliary obstruction, the bile quantity may be greatly lessened,[25] is more than borne out by our findings. Yet though the bile bulk may dwindle for a time nearly to zero, the secretion of pigment continues at the normal rate.

Lack of bodily activity, or, to put the matter positively, a sedentary life, is held to be contributory to cholelithiasis in many human instances. It supposedly leads to a stagnation of bile, to prevent which patients are ordered exercise. Yet exercise, as the present experiments show, does not act to increase the bile quantity. Perhaps it accomplishes good by lessening the number of hours a day during which postures are maintained that may be attended by temporary biliary obstruction, with the evils consequent thereon.[19]

The influence of fasting to lessen bile secretion has frequently been emphasized in the past; but for practical purposes the emphasis should be placed on the obverse side of the phenomenon, that is to say on the effect of certain foods, notably meats, to induce a copious bile flow, and on the general importance of a frequent food intake if the bile is to be kept moving properly. We have often had occasion to note that one of the best of cholagogues, and a normal one, the bile itself, may fail to cause any considerable increase in the rate of flow when fed in large quantity, if the animal be eating poorly, though in the same dog under better conditions, the same material will give marked results. This being true it is not surprising to find, as we have further, that when disease or warm weather has caused a lessening in the flow, bile feeding often increases it little if at all. The unsatisfactory effects of flogging therapeutically a jaded organ receives in these facts a new illustration.

By contrast, as showing the happy natural solution of a problem in therapeutics, the course of events when a temporary biliary obstruction is relieved may be referred to. There is then an immediate need to rid the organism of retained bile constituents, and that is accomplished through the elaboration by the liver of a copious bile, one so much more abundant than the normal that it is thinner than this latter, despite its extra burden of solids, and less concentrated

[25] Rovsing, T., *Hospitalstid.*, 1915, lviii, 249, abstracted in *J. Am. Med. Assn.*, 1915, lxiv, 1460.

in bile pigment. Doubtless the bile salts, potent as cholagogues under experimental conditions, and largely retained in the organism during obstruction, are responsible for the cholorrhea. The changes in the bilirubin output during the period of readjustment suggest that the bile continues copious just so long as there is an accumulation of biliary constituents to be evacuated. Danger of a clogging of the passages during their removal is in this way avoided.

The marked rapidity with which bilirubinuria followed clamping of the drainage tube of our intubated animals, as compared with the delay following operative ligation of the ducts, calls for some comment. The difference is plainly due to the differing rates of bile secretion in the two cases. Afanassiew[26] has demonstrated that the delay in the appearance of jaundice after operation to close the common duct occurs because the dilated duct system serves as an emergency reservoir for bile. Only after this reservoir has been filled does the resorption of bile from the liver into the body fluids begin; and in freshly laparotomized animals, as we have found, the bile is scanty for some time, so the reservoir fills slowly. Thus it comes about that the jaundice is staved off for a longer period than after obstruction unaccompanied by operation in intubated dogs yielding a copious secretion. But even in these latter, as already mentioned, the bile yield of 12 hours may accumulate back of an obstruction without the development of bilirubinuria. It would be enlightening to know, in connection with day to day variations in the bilirubin output, the precise abilities of the liver to retain bile constituents within the parenchyma.

The changes in the bile that follow operation upon the ducts, more especially the viscid thickening of it, constitute a potentially serious menace of obstruction. Whether this menace ever asserts itself under clinical conditions we are not in a position to state. In one of our animals in which obstruction occurred from such cause, the tube through which the viscid bile had to flow was about 30 cm. long and only $2\frac{1}{2}$ mm. in diameter, that is to say much longer and narrower than is a distended common duct.

[26] Afanassiew, M., *Z. klin. Med.*, 1883, vi, 281.

SUMMARY.

By a method of permanent intubation the entire output of bile from dogs has been obtained in a sterile state over long periods of time, and studied quantitatively. The secretion of the first few days after the initial operation is scanty, contains more pigment than that secreted later, and is sometimes so viscid as to cause temporary obstruction within the collecting tube. The small amount and thick consistency of the fluid are referable to direct injury of the liver and ducts, while the abundant bilirubin is derived, in part at least, from the hemoglobin of extravasated blood. In some animals in which there was accidental mercuric chloride poisoning at the time of operation a suppression of the bile followed which, in one instance, was complete during the 48 hours before death. In a dog developing mixed infection of the biliary tract, an almost colorless fluid, glairy as white of egg, gradually took the place of the bile.

Not until a week or 10 days after operation does the bile acquire the character which it maintains later. The quantity of this later bile, as measured in successive 24 hour specimens, is greatly less than that recorded by previous investigators, a difference attributable to disturbing influences inevitable to the method of collection they employed. By our method, it averaged from 3.5 to 9.5 cc. per kilo of dog in 24 hours, though transient variations from 1 cc. up to 14 cc. were encountered. Some dogs give consistently far more bile than others. The frequently recorded effects of fasting to lessen the rate of secretion, and of a meat diet to increase it more than a carbohydrate one, were noted often. Contrary to expectation, vigorous exercise does not act to increase the quantity of the bile. During hot weather this may sink greatly, although the animal remains in good condition; and during intercurrent diseases unassociated with jaundice, the flow may almost cease. One of the best of cholagogues, bile by mouth, fails of effect under such circumstances. It acts best when the animal is healthy, the weather not oppressive, and the food intake abundant.

The bilirubin output, after the immediate effects of the operation have worn off, remains nearly constant from day to day, though often exhibiting slow, wave-like variations, each extending over a

week or more. These slow changes are synchronous with similar alterations in the hemoglobin percentage of the circulating blood. A recognition of them is important to studies of the bilirubin yield. A mild anemia, absent in controls maintained under like conditions, regularly develops in unexercised dogs completely deprived of bile, despite their apparent health, and as it does so the bilirubin output falls off. Prior to the development of the anemia the output averages about 7.5 mg. per kilo in 24 hours, a yield which closely corresponds with that recorded by previous workers. Vigorous exercise of animals previously sedentary causes an increased pigment output presumably as result of increased blood destruction.

Since the daily pigment output is approximately constant, whereas the fluid quantity undergoes frequent and great changes, it follows that the pigment concentration must vary inversely as the fluid quantity, and vary greatly. It does both. No matter how large or small the 24 hour specimen of bile may be within "normal" limits, therein will be found the customary quota of pigment. When, under pathological conditions, the bile flow almost ceases, the pigment concentration becomes extremely great. A similar reciprocal relationship between concentration and fluid quantity would seem to hold for the mucinous element of the bile. Scanty biles are ropy as a rule, and copious ones are watery.

Temporary obstruction was produced by clamping the outlet tube from the common duct, and the bile yield following upon its relief was studied. Secretion was for a time far more copious than usual, with a low pigment content per cubic centimeter, and tended to remain so until the accumulated pigment had been voided. As much extra bilirubin is put forth after 24 hours of obstruction as if the liver continued to manufacture the pigment without interruption during it. Obstruction for this length of time appears to cause no subjective disturbance in most instances, though bilirubinuria develops. Even this sign of bile retention may be lacking when obstruction endures but 12 hours.

The physiological and clinical significance of these facts is briefly discussed.

STUDIES ON THE TOTAL BILE.

II. The Relation of Carbohydrates to the Output of Bile Pigment.

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For two generations the belief has been general that the pigment of the bile is derived wholly from the pigment of destroyed blood; and of late years the quantitative relationship supposedly existing between the two has been utilized as a basis for calculations on the rate of normal blood destruction.[1] Recently, though, Hooper and Whipple[2] have published experiments which have convinced them that the bilirubin is normally formed in considerable part from the carbohydrates of the food. If such be the case, quantitative studies of the bilirubin output and of urobilin in the stools of patients with blood diseases lose largely in significance, as does also much of the accepted reasoning upon the course of normal blood destruction.

The findings of Hooper and Whipple are clear-cut. But they were obtained through quantitations on a rather small fraction of the 24 hour output of bile, and these authors assumed that the fraction was representative of the secretion in general. Is this assumption warranted? Pavy has described the condition of the liver after a meal rich in carbohydrates.[3] It becomes swollen with glycogen, large, pale, and friable, and the capillary spaces are so encroached upon by the parenchyma as to suggest the existence of an actual vascular

[1] Zoja, L., *Folia hæmatol.*, 1910, x, 232. Goodman, E. H., *Beitr. chem. Physiol. u. Path.*, 1907, ix, 91. Abderhalden, E., Lehrbuch der physiologischen Chemie, Berlin and Vienna, 4th edition, 1920, i, 746.

[2] Whipple, G. H., and Hooper, C. W., *Am. J. Physiol.*, 1916, xl, 349. Whipple, G. H., *Arch. Int. Med.*, 1922, xxix, 711; *Physiol. Rev.*, 1922, ii, 440.

[3] Pavy, F. W., The physiology of the carbohydrates; their application as food and relation to diabetes, London, 1894.

compression.[4] May it not be that the increased bilirubin output observed by Hooper and Whipple is a temporary result of these marked changes, produced perhaps by pressure or replacement, and later compensated for by a lessened output? A method recently devised for total bile collection over long periods of time has afforded us opportunity to test the point.

Hooper and Whipple employed dogs with open bile fistulas made according to the classical method of Schwann; and they collected the secretion during 6 or 8 hours of each 24, the specimen of the first 2 hours serving in some experiments as control to that of the later ones. Between whiles the bile was allowed to dribble away from the fistula opening and the dogs could lick it up at will. For some days before each test with carbohydrates a diet of cooked lean meat and liver was fed. The carbohydrate ration,—bread and milk with a small bone,—was then abruptly substituted, or else dextrose was given by stomach tube, or intravenously. A remarkable increase in the output of bilirubin occurred within the next few hours. As the authors say, "On a strict meat diet the bile pigment curve is at its lowest level, but with a sharp transition to a diet rich in carbohydrates, a rise of 50 to 100 per cent in bile pigment output may be noted,"[5] whence the conclusion that "the bile pigments can be modified at will by diet factors."[6]

The most interesting of the protocols are those dealing with the effects of food, since they are indicative of what may perhaps be expected under normal conditions. The direct injection of a dextrose solution into the circulation brings with it a number of extraneous possibilities which must be considered in any interpretation of the results. For these reasons, though we have made both feeding and injection experiments, special emphasis will be laid on the effects of food. The giving of dextrose solutions in large quantity by mouth we have seldom practised (Text-fig. 2, Dog 18; Text-fig. 4, Dog 17), since it frequently induces vomiting.

In our experiments the diets used, the strength and amounts of the dextrose solutions, and the methods in general, have been identical with those of Hooper and Whipple. The dextrose was in part Merck's "highest purity," in part the C. P. product of the Corn Products Refining Company. The water in which it was dissolved had been twice distilled. During a preliminary period the dogs re-

[4] Afanassiew, M., *Arch. ges. Physiol.*, 1883, xxx, 385.

[5] Whipple, G. H., and Hooper, C. W., *Am. J. Physiol.*, 1916–17, xlii, 256.

[6] Wisner, F. P., and Whipple, G. H., *Am. J. Physiol.*, 1922, lx, 119.

ceived cooked lean meat and liver (bullock's or sheep's) in the proportion of five to one. The authors just mentioned gave 200 gm. of liver, but as they do not record the amount of the mixture eaten each day this figure helps but little. After a few days on such diet the appetite of many of our animals flagged and the ultimate contrast in the effects of the diets resolved itself into one between fasting and carbohydrate administration. In certain instances all food was designedly withdrawn for a day or more (Text-figs. 1 and 2) prior to the giving of carbohydrates. The failure of appetite on the meat diet may have been largely due to the circumstance that the dogs were confined in metabolism cages, not exercised daily in a yard as was Hooper and Whipple's practise. Active exercise causes a considerable increase in the bilirubin output as measured in the 24 hour specimen,[7] thus introducing a factor of error that is better avoided. Despite the lack of exercise the animals remained in excellent condition. When the carbohydrate diet was given,—bread moistened with a little milk, and a small bone, as described by the authors just mentioned,—it was practically always taken greedily and in large amount. No better opportunity could have been desired for a demonstration of the influence of carbohydrates. The character and amount of the ingested food find record in the charts (Text-figs. 1 to 6) which serve the purpose of protocols.

The dogs yielded sterile bile to the collecting bag, or one infected in pure culture with an organism harmless both to the animal, as the eventual autopsy proved, and to the bile pigment, as shown by incubation tests with specimens of the same bile and of sterile biles inoculated from it. The 24 hour specimen was always so slightly infected as to appear water-clear. Its pigment content was determined by Hooper and Whipple's modification of the Salkowski reaction, the method used by these authors. A mixture of copper sulfate and potassium bichromate served as the color standard.[7] Two determinations were made on each bile specimen, as in our previous observations.[8]

The urine was followed in every instance for bilirubin (Gmelin reaction, the Salkowski quantitative method). In one animal, sufficient of the pigment sometimes appeared for quantitation (Dog 19, Text-figs. 1 and 2), as is not infrequently the case in apparently healthy animals; but in the others the urine was negative practically throughout. One of the criteria employed in the selection of the dogs was freedom from such "physiological" bilirubinuria. The hemoglobin percentage of the blood was followed by the Newcomer method. Not infrequently it underwent gradual changes during the period of observation, and the changes were usually accompanied by similar fluctuations in the bilirubin yield.[8] Superimposed upon these fluctuations were the day to day changes in the bilirubin, which are relatively slight under ordinary circumstances.

In many of the animals injected with dextrose solution (Text-figs. 4 to 6) a 6 hour collection of bile, corresponding with that of Hooper and Whipple, was

[7] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 47.

[8] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 395.

made each day, followed by an 18 hour collection. As the charts show, the output of pigment during the shorter period furnishes but a poor index to that of the entire 24 hours. This latter is, as just mentioned, almost constant from day to day under ordinary circumstances. For these reasons 24 hour specimens have been preferred for most of the work.

The charts (Text-figs. 1 to 6) suffice to record the results. It will be seen from them that the ingestion of carbohydrate food in large quantity after a diet of cooked lean meat mixed with liver is wholly devoid of effect on the bilirubin output in the 24 hour bile specimen. It may be objected that our animals, unlike those of Hooper and Whipple, were for some reason incapable of responding properly to the administration of carbohydrates; but they differed from those of the observers mentioned only in that all of the bile was studied instead of a small fraction, that the animals had no open wound, that some of them were mildly anemic, and that the potentially confusing influences of pathogenic infection of the biliary tract, of yard exercise, and of the licking up of bile, were avoided. In certain cases in which a 6 hour specimen was taken separately, a transient hastening in the rate of secretion of bilirubin occurred, giving the effect of a rise in output of the sort recorded by Hooper and Whipple (Text-figs. 4 to 6),—conclusive evidence that the results of our experiments may properly be compared with those of the observers mentioned.

In all save one instance (Text-fig. 6, second injection), in which the dextrose injection was followed by a rise in the bilirubin content of the 6 hour specimen, there was a compensatory drop in the output during the succeeding 18 hours, and the total for the combined periods merely amounted to that of control days. This instance is exceptional in other ways. At the time of its occurrence, the animal was in poor condition, as result of chronic intestinal obstruction, markedly anemic, and showed sudden great fluctuations in the hemoglobin percentage of the blood, and as great ones in the bilirubin output. The red cells of some dogs are so fragile[9] that it would not be surprising to find them injured by intravenous injections of any non-colloid solution.

Comment.

The data here presented would seem to prove that the rise in bilirubin output which follows the administration of carbohydrates is not due to an increase in the amount of the substance manufactured

[9] Rous, P., and Turner, J. R., *J. Exp. Med.*, 1916, xxiii, 219.

by the body, but merely to a temporary hastening in its evacuation. Whether this hastening is consequent on the deposition of glycogen in the liver, an explanation which is suggested by the condition of the organ, has not been determined. Hooper and Whipple make a statement in connection with their 6 hour observations which may bear on the point. They state that: "The diet rich in carbohydrates may give a very high initial curve (perhaps double normal), which is apt to fall during succeeding weeks, but always remains somewhat above the mean curve." This is to say that the changes were much more marked when glycogen was first supplied in quantity to the liver than later when the organ contained the substance in considerable amount from day to day. Furthermore, "there is a tendency for the high bile pigment curve of a carbohydrate diet to approach the mean curve of a mixed diet. Also there is the same tendency for the low bile pigment curve of a meat diet to approach the mean curve of a mixed diet."[2]

Dextrose injections do not cause any consistent alteration in the rate of bile flow, which furthermore is almost regularly less on a carbohydrate diet than on one of meat. The increase in the pigment output can scarcely be laid then to a mechanical flushing out of the liver.

The present experiments have been the necessary first step toward a study of the quantitative relationship between blood destruction and the bilirubin output. It is obviously essential to know in this connection whether bile pigment can be derived from the food. In addition to carbohydrate food, as described, we have fed several diets consisting predominantly of protein, or of mixtures of protein and carbohydrate, all having the character in common that they are poor in fats. None has influenced the 24 hour output of bilirubin; and in this particular our work corroborates that of Hooper and Whipple with 6 hour specimens. Since such is the case no detailed records need be given.

CONCLUSION.

Carbohydrate feeding or injection produces often a temporary increase in the rate at which bilirubin is put forth in the bile, but none in the amount of the pigment secreted from day to day. There would appear to be no ground for the supposition that bilirubin is normally derived in part from the carbohydrates of the food.

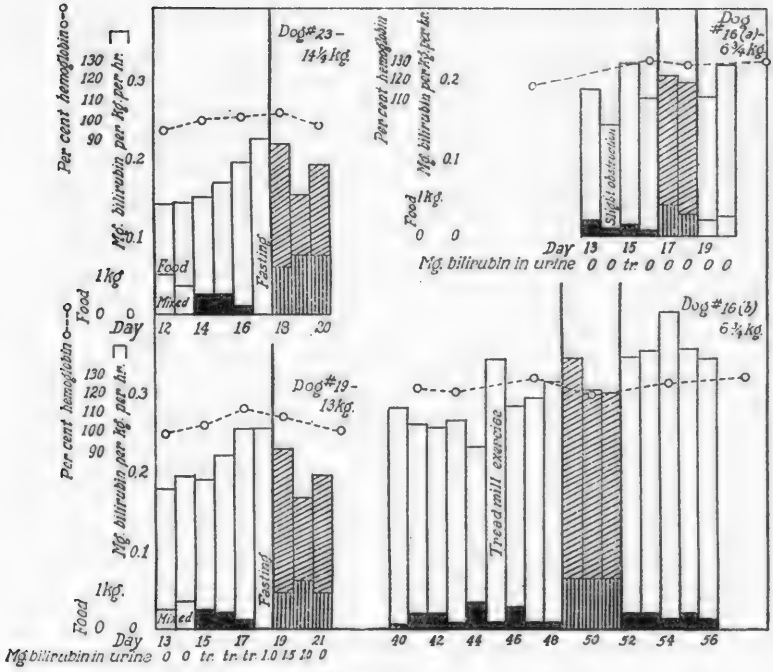
Explanation of Charts.

The bilirubin yield in the bile from day to day, the amount of food taken, and the hemoglobin percentage of the blood are recorded. A black column for the food indicates that it consisted of meat and liver, one vertically lined that bread, milk, and bones were given. For purposes of contrast a heavy vertical line has in some instances been placed to indicate the point at which carbohydrate feeding was begun; and slanting lines have everywhere been drawn across the bilirubin columns for the days upon which carbohydrate was given. In several instances in which 6 and 18 hour collections were made the total bilirubin yield for the combined periods has been indicated by means of a dotted horizontal line. In Text-figs. 4 to 6 the rate of bile flow is recorded. Notes on bilirubinuria are appended for every experiment in which a positive Gmelin reaction was obtained.

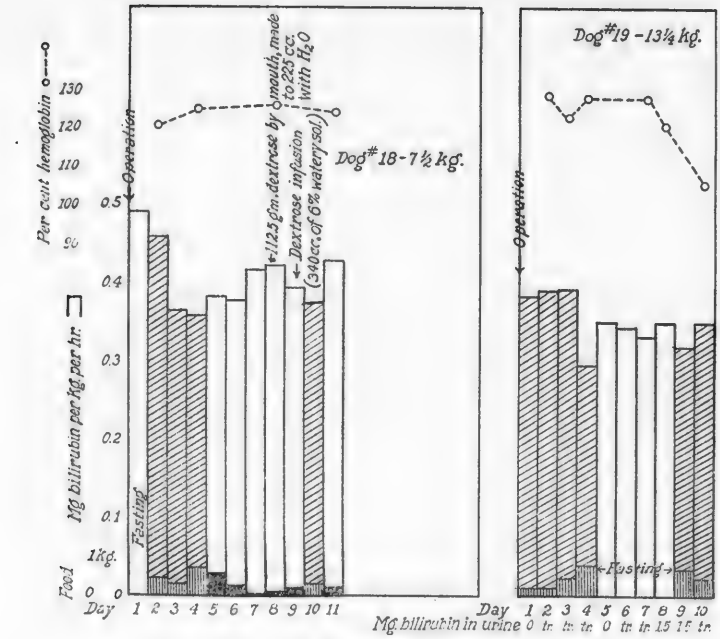
Legend for Text-Fig. 6.

This record should be considered apart from the others owing to the fact that the animal was in good condition only during the early days. It gradually lost weight and strength, because of a chronic obstruction of the small bowel by adhesions, as the autopsy showed. There were loss of appetite, anemia, large fluctuations in the hemoglobin percentage with corresponding ones in the bilirubin output, and, toward the close of the observations, diarrhea, a great drop in hemoglobin, and profound lethargy.

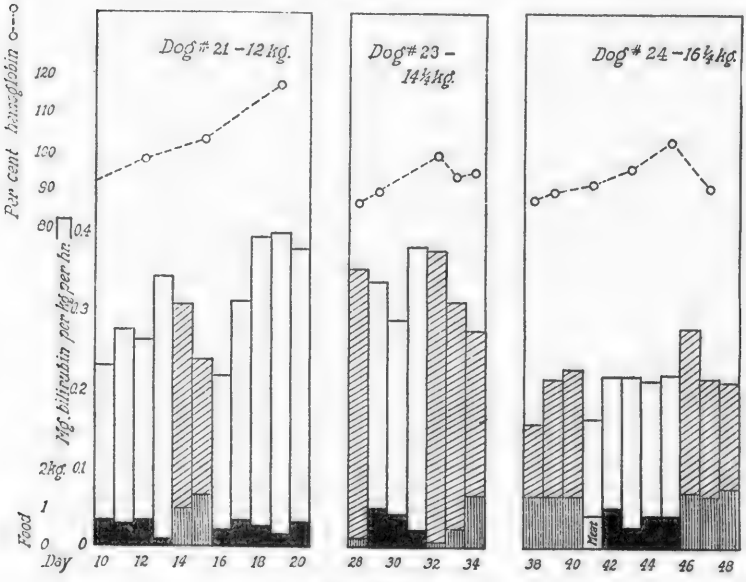
When the first dextrose injection and carbohydrate feeding were carried out the condition of the dog was good. It will be seen that they were without effect on the 24 hour output of bilirubin, though increasing the 6 hour output. The pronounced temporary rise in both outputs after the second dextrose injection occurred at a time when the signs of intestinal obstruction were marked and the condition of the dog was very abnormal. The record has been included for the sake of completeness.


Dog #23 – 14¼ kg.
Dog #19 – 13 kg.
Dog #16 (a) – 6¾ kg.
Dog #16 (b) 6¾ kg.
Per cent hemoglobin
Mg. bilirubin per kg. per hr.
Food
Mixed
Fasting
Slight obstruction
Treadmill exercise
Day
Mg. bilirubin in urine

TEXT-FIG. 1.


Per cent hemoglobin
Mg bilirubin per kg per hr.
Food
Day
Operation
Fasting
1 Kg.
Dog # 18 - 7½ kg.
+ 1/2 5 gm. dextrose by mouth, made to 225 cc. with H2O
← Dextrose infusion (340 cc. of 6% watery sol.)
Dog # 19 - 13¼ kg.
Mg bilirubin in urine 0 tr. tr. tr. 0 tr. tr. 15 15 tr.

TEXT-FIG. 2.



TEXT-FIG. 3.

Dog # 16 (a) – 6 ¾ kg.
Cc. bile per hr.
Per cent hemoglobin
Mg. bilirubin per kg per hr.
← Dextrose by mouth; vomited
← Dextrose infusion (320 cc. of 6% watery sol.)
Food
2 kg.
1
0
Day
20
21
22
23
24
25
26
27
28
29
Dog # 17 – 11¼ kg.
– 60 gm. cane sugar in 200 cc. H_2O by mouth
← 820 cc. of 5% dextrose in 0.7% salt sol. (infusion)
1 Kg.
7
8
9
10
11

TEXT-FIG. 4.

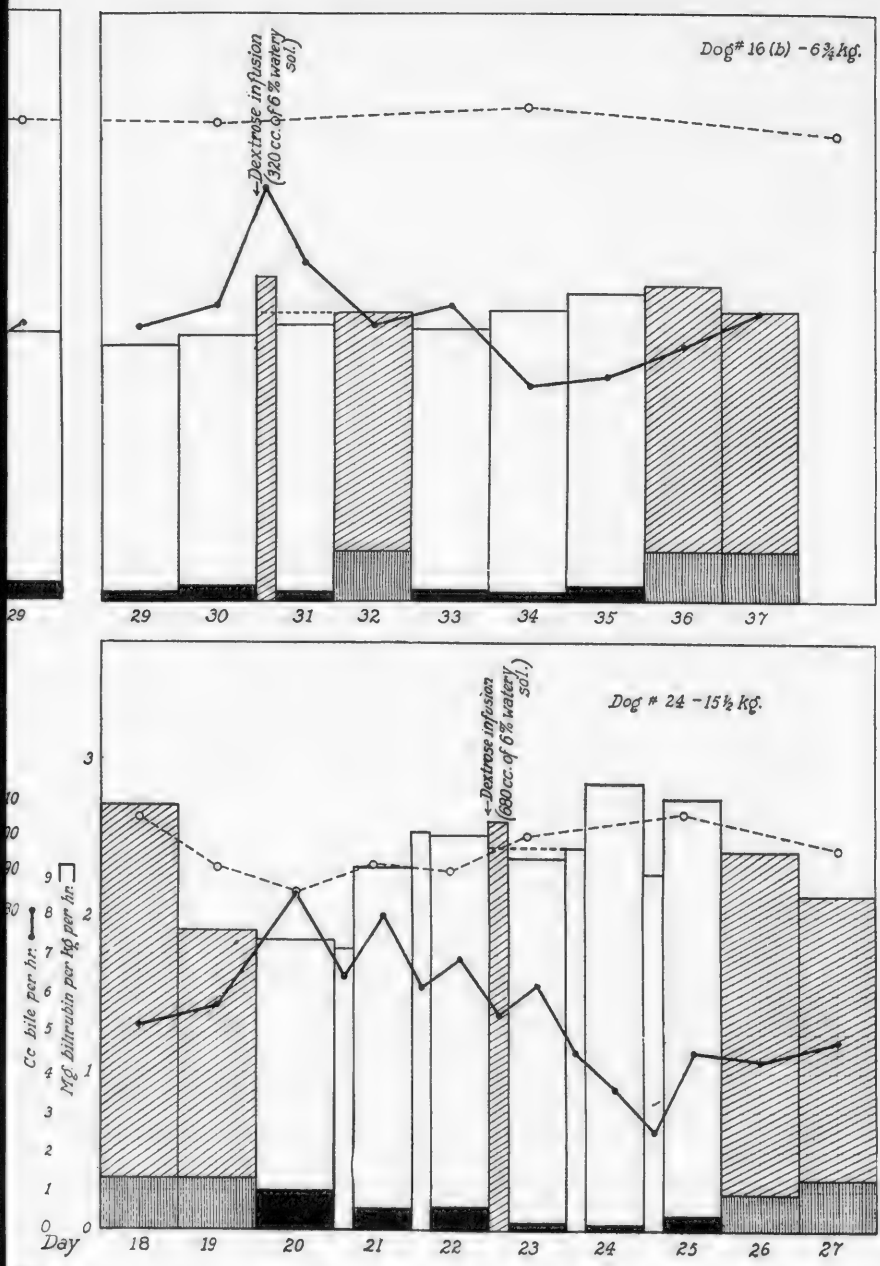
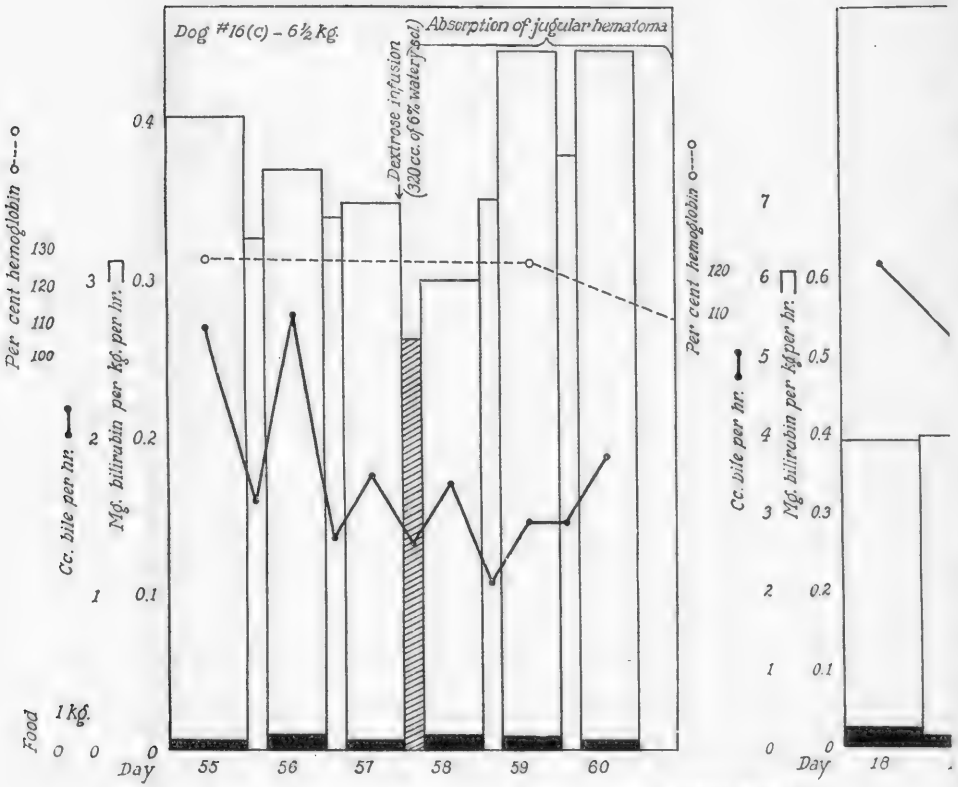

Dog # 16 (b) - 6¾ Kg.
←Dextrose infusion (320 cc. of 6% watery sol.)
29
30
31
32
33
34
35
36
37
Dog # 24 - 15½ Kg.
←Dextrose infusion (680 cc. of 6% watery sol.)
Cc bile per hr.
Mg bilirubin per Kg. per hr.
Day
18
19
20
21
22
23
24
25
26
27

FIG. 4.


Dog #16(c) – 6½ kg.
Absorption of jugular hematoma
Dextrose infusion (330 cc. of 6% water sol.)
Per cent hemoglobin
Cc. bile per hr.
Mg. bilirubin per kg. per hr.
Food 1 kg.
Day
55
56
57
58
59
60
18

TEXT-FIG. 5.

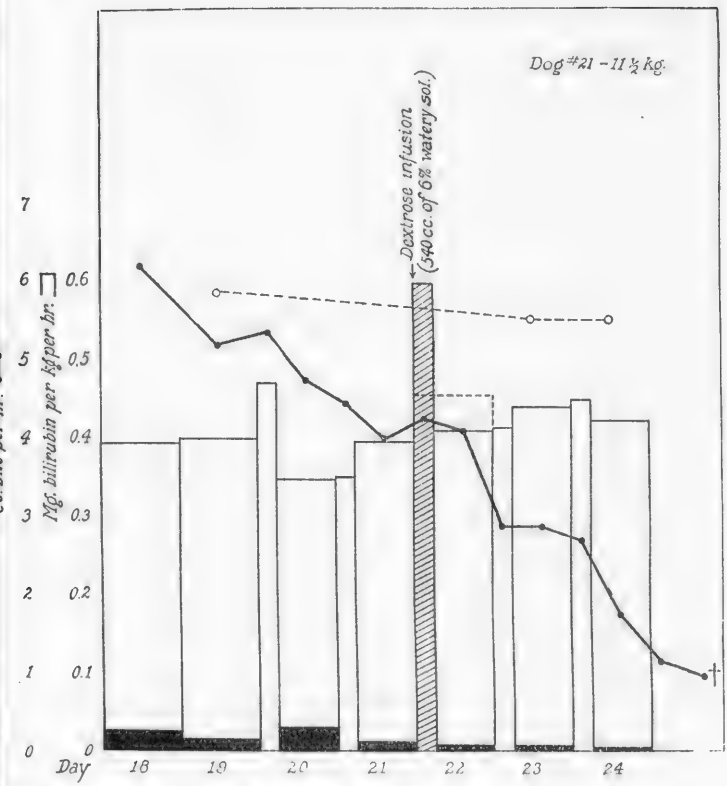
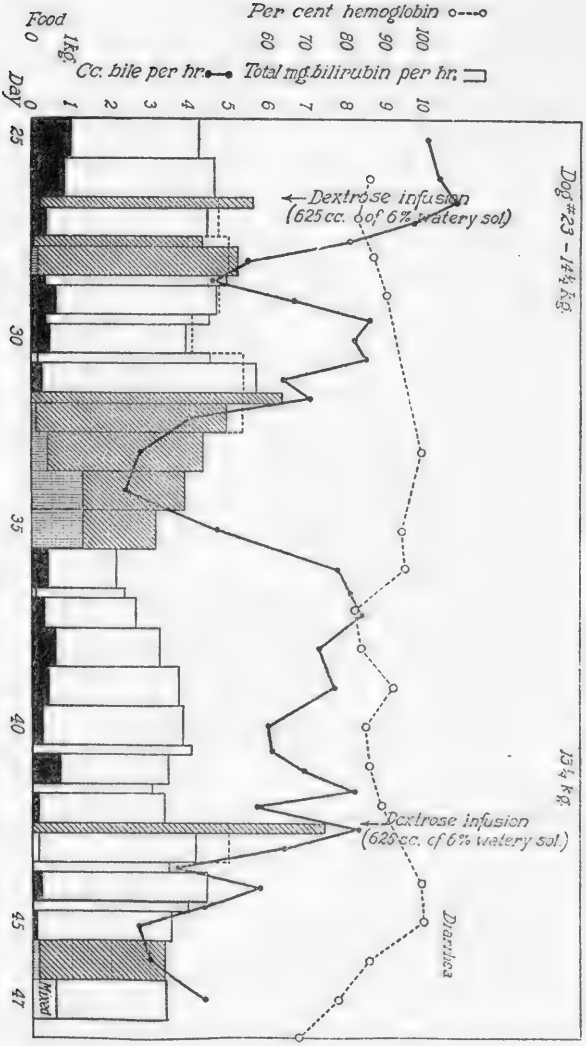

Dog #21 – 11½ kg.
← Dextrose infusion (340 cc. of 6% watery sol.)
Mg. bilirubin per kg per hr.
7
6
5
4
3
2
1
0
0.6
0.5
0.4
0.3
0.2
0.1
0
Day
18
19
20
21
22
23
24
†

FIG. 5.


Food
Per cent hemoglobin
Cc. bile per hr.
Total mg. bilirubin per hr.
Day
Dog #23 – 14½ kg.
Dextrose infusion
(625 cc. of 6% watery sol.)
15¼ kg.
Dextrose infusion
(625 cc. of 6% watery sol.)
Diarrhea
Mucus

TEXT-FIG. 6.

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